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UMI

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VOL. XV DECEMBER, 1961 NO. 4

A STUDY OF CANCER MORTALITY IN FARMING, QUARRYING, MINING AND OTHER OCCUPATIONS IN NORTH WALES AND CHESHIRE

P. STOCKS

From the Department of Biochemistry and Soil Science, School of Agriculture, University College of North Wales, Bangor

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In the course of a survey during 1952–56 of the environmental histories of residents in the counties of Anglesey, Caernarvon, Denbigh, Flint and Merioneth, and in the parts of Cheshire and Lancashire included in Liverpool Hospital Region who died of cancer, information was collected as to the occupations followed, kinds of houses lived in and sources of vegetables during 20 years preceding the onset of illness (Stocks, 1958). Although the occupational histories were coded, no analysis of them could be made for the survey report, but a limited study has now been effected in respect of the Welsh counties and of Cheshire excluding the county boroughs in order to find whether men whose occupations must have brought them into continual contact with the soil were more liable than other men to cancer of the stomach in those parts of North Wales where the incidence of that form of cancer is peculiarly high.

It has been found that in the areas with high incidence there is a statistical association between frequency of occurrence of gastric cancer in houses and certain characters of the soil around them (Stocks and Davies, 1960), and if this is due to the presence of some carcinogenic agent it might reach the stomach through food which was either itself grown in the soil or contaminated by the hands of men in continual contact with soil, other possible channels being the water supply or radiation. If actual and frequent contact with the local soil is an important factor it would be expected that (*a*) men who had been engaged in farming, quarrying and mining occupations would have higher death rates from stomach cancer than other men of the same ages in North Wales whereas no such difference would appear in Cheshire ; (*b*) the wives of men in those occupations would not show a corresponding excess over other wives ; (*c*) living in houses having a vegetable garden and habitually consuming food from the garden by men who had not been engaged in those occupations and by women could fail to show any statistical association with mortality from stomach cancer. The main purpose of this enquiry has been to discover which if any of these conditions were satisfied.

Occupational analysis by proportionate mortality

The 2558 deaths of men aged 20 and over from cancer in the six county areas were classified according to the primary cancer site (oesophagus, stomach, intestine,

rectum, lung and bronchus, other) and grouped by county of last residence and by occupational history as follows :

Farming or agricultural work (codes 010–030) at some time but no record of work in quarries ;
Quarry or mine work other than coal (codes 051–059) at some time ;
Coal mining (codes 041–049) at some time but not farm or quarry work ;
Other occupations with no record of any of above.

Table I shows the resulting distribution of deaths and expresses the frequencies of cancers of each primary site as percentages of all cancer. Before evaluating age-adjusted death rates for more exact comparisons the crude site proportions in the special occupational groups will be compared with those in the residual group of other occupations.

In Caernarvonshire and Merioneth standardized mortality of men from stomach cancer during 1947–55 was just twice the national rural average, and for oesophageal cancer during 1950–55 the corresponding ratios were 1·8 and 1·5 in the two counties. Over most of this area incidence of stomach cancer tends to be higher in houses where the garden soil contains more organic carbon or zinc. The counties are largely pastoral and there are extensive slate quarries whilst igneous rock is quarried at several places. It appears from Table I that among the 440 men who died without record of work at any time in farm, quarry or mine the stomach was the primary site in 26·4 per cent, oesophagus in 6·1 and lung in 18·0 per cent. In the farm group of 112 men the corresponding proportions were 44·7, 1·8 and 5·4, showing pronounced excess of stomach ($18{\cdot}3 \pm 5{\cdot}2$) and of stomach and oesophagus categories combined ($14{\cdot}0 \pm 5{\cdot}2$), and a pronounced deficiency of lung and bronchus ($12{\cdot}6 \pm 2{\cdot}8$). In the quarry group of 98 men the proportions were stomach 35·7, oesophagus 6·1 and lung 24·5 per cent, showing an excess of stomach ($9{\cdot}3 \pm 5{\cdot}3$), of stomach and oesophagus combined ($9{\cdot}3 \pm 5{\cdot}0$) and also of lung ($6{\cdot}5 \pm 4{\cdot}7$).

Employment in the quarries has been declining and was recorded as the last occupation before death or retirement for only 74 of the men, with site percentages of stomach 34, oesophagus 7, intestine and rectum 16, lung 23 and others 20. When mortality studies have to be based on the last occupation, which is usually the only information available, delayed effects of occupation on cause of death must be partly obscured by transfer to another group of men who had changed their occupation. In this series classification by last occupation gave quarrymen excesses over the residual group of only 5·8 and 5·1 in the percentages of stomach and lung cancer whereas classification based on all occupations during the last 20 years gave excess of 9·3 and 6·5 respectively. Similarly for farming, classification by last occupation alone reduced the excess in stomach percentage from 18·3 to 14·0. This illustrates the fact that in searching for evidence of delayed occupational effects it is worth while recording and using the whole occupational history in preference to the last occupation only.

In Denbighshire and Flintshire standardized mortality of men from cancer of the stomach, and also of the oesophagus, is about $1\frac{1}{2}$ times the national rural average. The ground is more arable and in the carboniferous strata the quarries are of limestone or sandstone rather than of igneous rock and there is a coal field employing over 6000 men. Amongst the 679 men who died of cancer in the two counties with no record of having worked in farm, quarry or mine the stomach

TABLE I.—*Deaths of Men from Cancer in North Wales and Cheshire from July, 1952 to June, 1955, distinguishing those whose occupation had at any time been Farming (codes 010–030), Quarry or Mine Work, not in Coal (codes 051–059) and Coal Mining (codes 041–049)**

Primary cancer site	Numbers of deaths					Percent of all cancer			
	Farm	Quarry	Coal	Other	Total	Farm	Quarry	Coal	Other
Caernarvonshire and Merioneth									
Lung	6	24	1	79	110	5·4	24·5	—	18·0
Stomach	50	35	1	116	202	44·7	35·7	—	26·4
Oesophagus	2	6	1	27	36	1·8	6·1	—	6·1
Intestine	14	9	2	56	81	12·5	9·2	—	12·7
Rectum	7	5	1	36	49	6·2	5·1	—	8·2
Others	33	19	1	126	179	29·4	19·4	—	28·6
All cancer	112	98	7	440	657	100	100	—	100
Denbighshire and Flintshire									
Lung	17	8	33	152	210	11·5	25·0	24·1	22·4
Stomach	46	6	33	146	231	31·1	18·8	24·1	21·5
Oesophagus	9	1	8	28	46	6·1	3·1	5·8	4·1
Intestine	18	—	6	77	101	12·1	—	4·4	11·3
Rectum	12	3	15	60	90	8·1	9·4	10·9	8·8
Others	46	14	42	216	318	31·1	43·7	30·7	31·9
All cancer	148	32	137	679	996	100	100	100	100
Anglesey									
Lung	10	2	—	23	35	16·7	—	—	18·7
Stomach	14	3	1	24	42	23·3	—	—	19·5
Oesophagus	4	—	—	3	7	6·7	—	—	2·4
Intestine	9	1	—	21	31	15·0	—	—	17·1
Rectum	5	1	—	10	16	8·3	—	—	8·1
Others	18	2	1	42	63	30·0	—	—	34·2
All cancer	60	9	2	123	194	100	—	—	100
Cheshire†									
Lung	16	—	2	174	192	16·2	—	—	28·8
Stomach	20	—	1	127	148	20·2	—	—	21·0
Oesophagus	6	—	—	14	20	6·1	—	—	2·3
Intestine	11	—	1	58	70	11·1	—	—	9·6
Rectum	5	—	1	43	49	5·0	—	—	7·1
Others	41	—	2	189	232	41·4	—	—	31·2
All cancer	99	—	7	605	711	100	—	—	100

* If occupations in more than one group had been followed the order of precedence was quarry, farm, coal.

† County districts in the Liverpool Hospital Region (western part of Cheshire).

was primarily affected in 21·5 per cent, oesophagus in 4·1 and lung in 22·4 per cent. In the farm group of 148 men the corresponding proportions were 31·1, 6·1 and 11·5 respectively, showing as before a pronounced excess of stomach ($9·6 \pm 4·1$) and of stomach and oesophagus categories combined ($11·6 \pm 4·3$) and a pronounced deficiency of lung cancer ($10·9 \pm 3·1$). Those who had worked in the quarries showed no such tendency to gastric cancer as was found for the slate and stone quarry workers in Caernarvon and Merioneth, and this agrees

with a finding in the occupational mortality figures for England and Wales in 1930–32 (Registrar General, 1938) which showed the following numbers of deaths of miners and quarriers at ages 20–65, classified according to the kind of rock :

		All cancer	18, stomach	9
Group No. 135	Ingneous rock			
139	Slate, Caernarvonshire	,, 24	,,	11
140	Slate, elsewhere	,, 13	,,	7
136	Limestone	,, 30	,,	7
137	Sandstone	,, 20	,,	4

In the igneous rock and slate workers 49 per cent of cancers were of the stomach, compared with 22 per cent in the limestone and sandstone groups. In respect to the coal miners in Denbighshire, Table I shows an excess of only 4·3 in the proportion of stomach and oesophagus compared with the residual group, but it will be seen below that their age-adjusted death rate for stomach cancer was similar to that of the farm group and significantly above that of the residual occupations.

In Anglesey male mortality in terms of the national rural average is about 1·8 for stomach cancer and incidence is correlated with certain soil characters (Davies and Wynne Griffith, 1954). The geology is complex, partly pre-Cambrian, the soil is arable and about a quarter of the men are engaged in agriculture. Amongst the 123 men who died of cancer with no record of farm, quarry or mine work the stomach was the primary site in 19·5 per cent, oesophagus in 2·4 and lung in 18·7 per cent. In the farm group of 60 the proportions were 23·3, 6·7 and 16·7, and in the small group of 11 who had worked in quarry or mine they were 36, 0 and 18, the excess of stomach and oesophagus categories combined being of the same kind as in the counties of Caernarvon and Merioneth.

In the parts of Cheshire covered by this study mortality from cancer of the stomach is not abnormal, and no relation has been found with organic carbon in the soil although there appears to be a correlation with zinc content in the garden soils. Amongst the 605 men who died of cancer with no record of having worked in farm, quarry or mine the primary site was stomach in 21·0 per cent, oesophagus in 2·3 and lung or bronchus in 28·8 per cent ; and amongst 99 who had worked on farms the proportions were 20·2, 6·1 and 16·2 per cent respectively. The farm group showed, therefore, a smaller percentage of lung than the residual group (difference $12{\cdot}6 \pm 5{\cdot}6$) but, in contrast with the Welsh counties, no excess of stomach cancer.

Evaluation of age-adjusted death rates

The population of men at the census is classifiable according to occupation then if still working or just before retirement if not, and no information is obtained as to previous employment. Furthermore in respect of separate counties the numbers of retired men are not analysed according to occupation before retirement. In Caernarvonshire at 1951 census there were 40,734 men aged 20 and over of whom 5387 were then in agricultural work and 3404 in quarry work, 9 were coal miners and 25,084 in other occupations, leaving 6850 retired who could not be distributed from available tabulations. Populations at risk for the deaths in Table I cannot be derived, therefore, from the county census and an indirect method of estimation has to be used to calculate death rates.

Standardised death rates of males from total cancer excluding the stomach, lung and bronchus show no appreciable variation in England and Wales attributable to geography although they are affected to some extent by urbanisation and occupation. Thus in 1954 the arithmetic mean of the standardised rates at ages 35 and over in rural districts and small urban areas were 227, 220, 216 and 226 per 100,000 in the North, Midland and East, South and Welsh regions respectively. The areas covered by the present study have no large towns and it can be assumed that after correction for differences in age distribution between the counties the rate for cancer excluding stomach and lung is constant within each of the occupational groups used in the study. If R is the death rate from this cause at ages 20 and over amongst the farm workers in England and Wales as a whole, the death rate in any one of these areas adjusted for age to the national rural population as standard will also be R. If the age-specific rates of the farm workers produce a rate S when applied to the standard population and a rate C when applied to the county population, then the crude death rate at ages 20 and over will be RC/S, and if there were d deaths of farm workers in the county from cancer excluding stomach and lung the population of such workers in the county can be estimated as dS/CR*. For coal miners and quarry workers the same method can be used to estimate the local populations; and for the residual group of all other occupations the appropriate basic population for calculating S is that of all males since these occupations are both urban and rural for the most part.

If this procedure is repeated for cancer of a specific site such as the stomach and the deaths in the county within one of the occupation groups, numbering d', are corrected by the resulting factor S'/C', the death rate from that cause, age-adjusted to all men in that occupation in England and Wales, will be $d'S'/C'$ divided by the estimated population dS/CR, that is to say Rfd'/d where f is the correcting factor $S'C'/C'S$. For cancer of the stomach the factor f approximates closely to unity owing to the similarity between the progression on age for stomach cancer mortality and that for all cancer excluding stomach and lung, and if population estimates are not required the local death rate, age-adjusted to all males in the occupation in question, is given approximately by Rd'/d. For cancer of the lung and some other sites however the correcting factor f may differ appreciably from unity.

Table II shows the mean annual death rates at 20 and over in 1949–53 from all cancer excluding stomach and lung amongst men in the four occupational groups, the factors C/S to adjust these for age in order to estimate the populations in four county areas, and the correcting factors f to apply to the ratios d'/d between the deaths from stomach and lung cancers to those from all cancer except stomach and lung in order to obtain areal rates age-adjusted within each occupational group. Table III gives the values of d'/d derived from the observed frequencies in Table I, and the death rates derived from these by the formula which are comparable within each column; but since each is based on a national

* In Caernarvonshire and Merioneth the annual value of d for farm workers by last occupation was 23·7 and the correction factor C/S was 1·13, and from the national statistics for 1949–53 R is 2·06 per 1000, leading to a population estimate of 10,200. The census of 1951 showed there were 8456 men in the agricultural groups at ages 20 and over out of 45,884 still working, and there were 8927 no longer occupied whose work before retirement could not be ascertained from the existing tabulation, but about one fifth of these had probably been in the farm group, and the estimated total is reasonable.

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TABLE II.—*Death Rates per* 100,000 *Men aged* 20 *and over in Four Occupation Groups from Cancer excluding stomach and lung, and Areal factors for calculating age-adjusted rates within each occupation for cancers of the Stomach and Lung*

	Farm workers	Quarry workers	Coal miners	Other occupations
	All cancer except of stomach and lung			
National death rates (R)	206·3	183·0	183·5	163·9
Age-adjusting factor C/C for:				
Caernarvon & Merioneth	1·13	1·12	—	1·20
Denbigh & Flint	1·00	1·13	0·98	1·07
Anglesey	1·13	0·98	—	1·20
Cheshire*	0·94	—	—	1·00

	Cancers of Stomach and Lung							
	Stomach	Lung	Stomach	Lung	Stomach	Lung	Stomach	Lung
Correcting factor f for:								
Caernarvon & Merioneth	1·00	1·12	0·99	1·12	—	—	1·01	1·13
Denbigh & Flint	0·99	0·95	1·01	1·08	1·04	1·02	1·01	1·04
Anglesey	1·00	1·08	1·04	1·02	—	—	1·02	1·08
Cheshire*	1·03	0·95	—	—	—	—	0·99	1·02

* Part of county in Liverpool Hospital Region excluding county boroughs.

standard for the occupation in question and the standards are not quite the same these rates are not comparable between one column and another.

TABLE III.—*Estimated Death Rates per* 100,000 *Men aged* 20 *and over according to their past occupations, adjusted for age differences within the occupation*

County area	Farm workers		Quarry workers		Coal miners		Other occupations	
	d'/d	Rate	d'/d	Rate	d'/d	Rate	d'/d	Rate
	Cancer of Stomach							
Caernarvon & Merioneth	0·893	184	0·897	163	—	—	0·473	79
Denbigh & Flint	0·541	111	0·333	64	0·465	110	0·383	63
Anglesey	0·389	80	0·75	139	—	—	0·316	53
Cheshire (survey area)	0·317	65	—	—	—	—	0·421	68
	Cancer of Lung							
Caernarvon & Merioneth	0·109	23	0·615	113	—	—	0·322	56
Denbigh & Flint	0·200	43	0·444	78	0·465	82	0·399	67
Anglesey	0·278	60	0·50	95	—	—	0·303	55
Cheshire (survey area)	0·254	52	—	—	—	—	0·572	91

In order to make valid comparisons between the occupational groups in a county area the rates in Table III need to be further adjusted for differences in age distribution of men in those groups. This has been done by applying age-specific death rates from stomach cancer in all males of England and Wales to the age distributions of the occupations at the census and dividing the death rate at 20 and over in all males by the resulting calculated rate in the occupation to obtain comparability factors. The age distributions of farm, quarry and coal workers were obtained from the national census populations (Registrar General, 1957) and for the residue of occupations they were derived from the whole county

populations by ages after deducting the proportions in the special groups as estimated from populations at ages 20–64 provided by the Registrar General. The rates in Table III were then multiplied by the appropriate comparability factors, giving the rates in Table IV which are age-adjusted in respect of both area and occupation. The same has been done for cancer of the lung and bronchus. The standard errors can be taken as the rate divided by $\sqrt{d}$.

TABLE IV.—*Estimated Death Rates of Men aged 20 and Over according to their past occupations, adjusted for Areal and Occupational Age differences*

	Rates per 100,000				Difference from "Other"		
	Farm	Quarry	Coal	Other	Farm	Quarry	Coal
				Cancer of Stomach			
Caernarvon & Merioneth	143	158	—	66	+77±20	+92±26	—
Denbigh & Flint	86	62	106	61	+27±10	+1	+45±13
Anglesey	62	134	—	46	+16±12	+88±67	—
Cheshire*	50	—	—	65	−15±7	—	—
				Cancer of Lung			
Caernarvon & Merioneth	27	117	—	49	−22±5	+68±19	—
Denbigh & Flint	51	81	85	67	−16±7	+14±19	+18±11
Anglesey	71	94	—	52	+19±13	+42±47	—
Cheshire*	61	—	—	93	−32±9	—	—

* Part of county in Liverpool Hospital Region excluding county boroughs.

Comparing the occupations, the rate differences between the special groups and the residual group are shown in the table with standard errors of the differences. These indicate *excess* with probabilities of 700 to 1 or more for stomach cancer in farm workers of Caernarvon, Merioneth, Denbigh and Flint, quarry workers of Caernarvon and Merioneth and coal miners of Denbigh and Flint, and for lung cancer in the quarry workers of Caernarvon and Merioneth. On the other hand they indicate *deficiency* above the conventional significance level for lung cancer in farm workers of Caernarvon, Merioneth, Denbigh, Flint and Cheshire. It is evident from this that men who had worked habitually with soil or coal, slate or igneous rock showed pronounced excess of stomach cancer rates compared with men who had not done so, the excess being highly significant for the farm and quarry groups in the Caernarvon-Merioneth area and for farm and coal mine groups in the Denbigh-Flint area, but absent in the limestone and sandstone quarry workers of the latter area. In Cheshire however, where incidence of the disease is normal, farm workers showed no excess over other occupations. Lung cancer rates on the other hand were lower amongst farm workers, the deficiency compared with other occupations being present and significant in all areas except Anglesey; but the quarry and mining groups showed somewhat higher rates than the residual group with a significant excess in the Caernarvon-Merioneth area where igneous rock and slate is quarried.

If the incidence of stomach cancer had been at the rates normal for rural areas and not affected by occupation the deaths of men in Caernarvon and Merioneth counties in the 3 years would have numbered 17 in the farm group, 12 in the quarry group and 72 in other occupations, instead of 50, 35 and 117 as in Table I, indicating that about 55 per cent of the total excess was accounted

for by the farm and quarry workers who comprised no more than a quarter of the men at risk. This suggests that the effect of soil on stomach cancer mortality of men in this area arises to an important extent through actual contact with the earth, and if that is so it would be expected that stomach cancer mortality of the wives of farm workers would not show any considerable difference from that of wives of other workers. This can be tested in respect of the wives of men in social subclass IV*a*, agricultural workers, since during the survey in 1952–55 deaths of married women from cancer were coded according to the husband's social class, and in Table V the cancer site frequencies are shown for the subclass IV*a*, the rest of classes III–V, and classes I–II. It is estimated that one eighth of the last of these groups were wives of farmers (codes 010, 011, 020) who were not distinguished from the rest of class II in the coding ; but the Table shows that stomach cancer formed a rather smaller proportion of total cancer both in groups IV*a* and I–II than in the large residual group consisting of the rest of III–V, and it must be concluded that wives of the whole farming population showed no excess in the proportion of stomach cancer compared with other married women. It follows from the ratios to all cancer except lung and stomach by the method used for men that the estimated crude death rates from stomach cancer in the 3 groups of Table V were about 44, 39 and 40 per 100,000 at ages 20 and over, the corresponding national rates being 31, 24 and 22, so the women's excess of this form of cancer in North Wales is evidently no more pronounced on farms than elsewhere.

TABLE V.—*Cancer Deaths in Wives of Agricultural Workers (IVa) and Other Social Groups of Married Women in North Wales.*

	Deaths in 3 years			Per cent of all cancer		
Primary site	IVa	Rest of III–V	I–II	IVa	Rest of III–V	I–II
Lung and bronchus	—	30	12	—	3·8	3·7
Stomach	12	172	62	20·3	21·5	18·7
Oesophagus	5	27	8	8·5	3·4	2·4
Intestine and rectum	13	162	65	22·0	20·2	19·6
Other cancer	29	409	184	49·2	51·1	55·6
Total	59	800	331	100	100	100

History of residence with garden

In the course of the 1952–55 survey questions were asked from relatives after deaths from cancer of certain sites, mainly the lung, stomach, intestine, rectum (males) and breast, as to the kinds of house in which the deceased person had lived during the last 20 years, and these could be classified by the code into house with a vegetable garden (denoted here as *VG*), house with a garden but not providing vegetables (denoted as *OG*) and place of residence with no garden (denoted as *NO*). A question was asked also as to whether green vegetables had been consumed habitually from such a garden. The numbers for which no answer could be obtained were small and have been excluded in calculating percentages in the three groups. In respect of lung, larynx, oesophagus, stomach, breast and rectum cancers the questions were asked throughout 3 years of the survey, whereas for intestine they were asked over a shorter period, so absolute

figures are not comparable between the site groups, but since each group consists of consecutive and unselected deaths the percentages with garden are comparable. In north-west Wales, which consists of Anglesey, Caernarvon and Merioneth, the records for men assigned to the VG category about 90 per cent of farm workers, 50 per cent of quarry workers and 40 per cent of others ; and amongst all women the proportion was 42 per cent in this area, 46 per cent in Denbigh and Flint and 35 per cent in Cheshire.

TABLE VI.—*Deaths from Cancer amongst Men who had not worked in Farm, Quarry or Mine, and amongst Women, analysed by history of Residence with a Vegetable or Other Garden*

			Males			Females				
			Lung	Stomach	Other cancer	Lung	Stomach	Intestine	Breast	Other cancer
Possession of a garden during last 5 years										
Wales N.W.:	Numbers with or without	*VG*	25	54	72	9	58	52	62	42
		OG	25	17	37	6	35	26	27	17
		NO	40	46	66	14	58	44	44	33
	Percent	*VG*	27·8	46·2	41·1	31·0	38·4	42·6	46·7	45·7
		VG + OG	55·6	60·7	62·3	51·7	61·6	63·9	66·9	64·1
Wales N.E.:	Numbers with or without	*VG*	51	48	111	8	72	49	56	41
		OG	38	38	48	7	42	29	43	25
		NO	35	32	47	7	40	16	35	23
	Percent	*VG*	44·7	40·7	53·9	36·4	46·8	52·1	41·8	46·1
		VG + OG	71·8	72·9	77·2	68·2	74·0	83·0	73·9	74·2
Cheshire:	Numbers with or without	*VG*	38	34	47	11	33	21	41	20
		OG	75	37	74	14	37	28	48	36
		NO	40	29	40	5	20	12	24	14
	Percent	*VG*	24·8	34·0	29·2	36·7	36·7	34·4	36·3	28·6
		VG + OG	73·9	71·0	75·2	83·3	77·8	80·3	78·8	80·0
Possession of a garden during 15 years before that										
Wales N.W.:	Numbers with or without	*VG*	20	46	64	13	59	52	59	41
		OG	26	20	32	6	34	25	26	14
		NO	40	51	73	13	56	41	42	34
	Percent	*VG*	23·3	39·3	37·9	40·6	39·6	44·1	46·5	46·1
		VG + OG	53·5	56·4	56·8	59·4	62·4	65·3	66·9	61·8
Wales N.E.:	Numbers with or without	*VG*	52	45	97	8	78	49	53	42
		OG	37	36	48	4	37	23	39	25
		NO	33	33	57	6	37	22	39	21
	Percent	*VG*	42·6	39·5	48·0	44·4	51·3	52·1	40·5	47·7
		VG + OG	73·0	71·1	71·8	66·7	75·7	76·6	70·2	76·1
Cheshire:	Numbers with or without	*VG*	34	31	39	12	32	19	40	20
		OG	73	34	69	11	34	31	41	35
		NO	41	33	50	5	21	11	30	13
	Percent	*VG*	23·0	31·6	24·7	42·9	36·8	31·1	36·0	30·3
		VG + OG	72·3	66·3	68·4	82·1	75·9	82·0	73·0	80·3

Table VI shows the numbers of deaths in categories *VG*, *OG* and *NO* and compares the proportions having had a vegetable garden (*VG*) and any garden (*VG + OG*) amongst men who had not worked in farm, quarry or mine, and amongst all women, according to the primary site of cancer causing death. In Wales N.W., the area of highest stomach cancer mortality, there are no significant differences between the frequencies of vegetable or any garden for stomach,

intestine, breast and other cancer except lung. Men who died of lung cancer showed peculiarly low proportions with a vegetable garden compared with the stomach and other groups, the *VG* percentages during the last 5 years before onset of the disease being 27·8 ± 4·7 compared with 46·2 ± 4·6 and 41·1 ± 3·7, and during 15 years before that 23·3 ± 4·7 compared with 39·3 ± 4·5 and 37·9 ± 3·7 ; but the proportions having had a garden of any kind showed only slight deficiences. Women who died of lung cancer also showed a lower *VG* proportion than the other groups during the last 5 years but owing to the small number of lung cancers the differences are below significance level. In Wales N.E. comprising Denbigh and Flint counties there are no significant differences between the cancer groups except that men with stomach cancer showed a low *VG* proportion during the last 5 years, 40·7 ± 4·5 compared with 44·7 for lung and 53·9 ± 3·5 for other cancer, but this was not true for women. In the Cheshire area covered by this study, on the other hand, the VG were rather higher for stomach than for other cancer in men but were the same for stomach as for breast in women.

TABLE VII.—*Proportions of Decedents as in Table VI reported to have consumed Vegetables from Own Garden in last* 10 *years*

Group	Wales N.W.		Wales N.E.		Cheshire	
	Number of deaths	Per cent affirmative	Number of deaths	Per cent affirmative	Number of deaths	Per cent affirmative
Males:						
Lung	73	23·7 ± 4·4	124	32·3 ± 4·2	154	20·1 ± 3·2
Stomach	121	34·3 ± 4·1	119	36·1 ± 4·4	98	28·6 ± 4·6
Other	162	30·9 ± 3·6	194	44·8 ± 3·6	158	30·4 ± 3·6
Females:						
Lung	29	37·9 ± 9·0	19	21·2 ± 9·3	30	30·0 ± 8·4
Stomach	152	29·6 ± 3·7	157	42·7 ± 3·9	86	26·8 ± 4·8
Intestine	124	30·6 ± 4·1	96	41·7 ± 5·2	57	24·6 ± 5·7
Breast	132	33·3 ± 4·1	132	38·6 ± 4·2	114	30·7 ± 4·3
Other	91	39·6 ± 5·1	92	43·5 ± 5·2	70	24·3 ± 5·1

Table VII shows the frequencies of definite answers to the question whether green vegetables had been consumed habitually from the garden during the last 10 years, and the proportions of affirmative replies are similar to but rather lower than the *VG* proportions in Table VI. In each area men with lung cancer show lower proportions than those with stomach or other cancer, the differences from the latter group being statistically significant in two areas, but the women do not show this. There are no consistent differences between stomach and intestine, breast or other cancers.

It would appear from these results that, whatever is the reason for the connection between stomach cancer incidence in houses and the quality of the soil around them it does not arise to any important extent from the eating of green vegetables grown in the garden, nor is the incidence greater in houses having a garden than in other dwellings provided that farmers and quarry workers are first excluded.

The low proportions of lung cancer decedents who had lived in houses with a vegetable garden might be due to larger numbers of them having lived in large

towns before residing in North Wales. From samples of hospital patients living in North Wales who were questioned about previous residence it appeared that 22 per cent of those with lung cancer had lived in large towns compared with 18 per cent of those with other kinds of cancer (Stocks, 1958, Table XIII), a difference which seems hardly adequate to account for the deficiencies in Tables VI and VII. Response to a question as to source of milk supply also showed that out of 215 men in North Wales who died of lung cancer and had not worked in farm, quarry or mine 36 per cent were said to have been supplied with milk from local farms, compared with 50 per cent of 228 who died of stomach cancer and 50 per cent of 338 who died of other cancer.

SUMMARY

In the north-western counties of Wales where mortality from cancer of the stomach is very high, men who had worked at any time during 20 years before death on farms or in quarries suffered much higher death rates from this cause in a three year period than those who had not been so employed, and more than half of the total excess was accounted for by these groups In Denbighshire and Flint where stomach cancer mortality is not quite so high men who had worked on farms or in coal mines showed a similar excess in comparison with other men. In western Cheshire excluding the county boroughs, where stomach cancer mortality is normal, no such difference appeared for men who had worked on farms. Wives of agricultural workers in North Wales also showed no appreciable excess in death rates compared with other married women.

Histories of having lived in a house with a vegetable garden and of having consumed green vegetables from it were not associated with any excess of mortality from stomach cancer in comparison with other cancers excluding the lung, either amongst men who had not worked on a farm or in a quarry or mines or amongst women generally. These findings suggest that the association between excessive stomach cancer incidence and certain soil characters which has been found in North Wales does not arise through the agency of green vegetables grown on the soil, and in the case of men actual contact with the earth in their daily work would seem to be a factor of importance.

Lung cancer mortality was notably lower amongst men who had worked on farms and amongst others who had lived in houses with a vegetable garden, but was enhanced in those who had quarried slate or igneous rock.

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HISTOLOGICAL TYPES IN PRIMARY LUNG CANCER: RELATIVE FREQUENCIES IN VARIOUS SAMPLES OF A NATIONAL LUNG CANCER MATERIAL

EINAR PEDERSEN

From The Cancer Registry of Norway, Norwegian Radium Hospital, Oslo

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In a series of papers during the last decade Kreyberg has discussed the histological types in primary lung cancer and has convincingly demonstrated the significance of careful histological classification in epidemiological studies of this disease (Kreyberg, 1952–1961). Distinguishing between, on the one hand, epidermoid and anaplastic small-cell carcinomas (Group I tumours) and, on the other hand, adenocarcinomas, bronchiolo-alveolar-cell carcinomas, carcinoids ("adenomas"), and mucous gland ("salivary gland") tumours (Group II tumours), he has found that these two groups of tumours differ fundamentally in their incidence pattern and in their association with certain environmental factors generally recognized as aetiologically related to lung cancer. In particular, his thorough epidemiological studies in Norway and his studies of materials from various countries differing widely in lung cancer mortality have with remarkable consistency indicated that:

(1) The observed increase in lung cancer in certain population groups in this century is essentially accounted for by tumours belonging to his Group I. In contrast, Group II tumours seem to have increased very little, probably not more than can reasonably be ascribed to improved case finding,

(2) in the present situation, the ratio between the number of Group I and Group II tumours in suitable lung cancer materials from various regions enables an estimate to be made of the incidence rates of lung cancer in those regions. The higher the ratio Group I/Group II, the higher the incidence rate.

However, in the course of his investigations Kreyberg has observed that the relative frequency of the various histological types is highly dependent on how the material used for histological typing is obtained. He has, in particular, shown that autopsy material tends to contain a higher proportion of Group II tumours than surgical material collected from the same geographical region, and he has repeatedly emphasized the necessity of basing comparisons between different regions or different time periods on materials of similar character (Kreyberg, 1959; Ferrari and Kreyberg, 1960; Kreyberg and Saxén, 1961).

Realizing that the material collected by a national cancer registry may provide an opportunity to study the extent and nature of selection in various samples of lung cancer material from a defined population—e.g. according to how the pathological specimen has been obtained—Kreyberg suggested that such a study be undertaken by the Cancer Registry of Norway. The following is an account of the findings.

MATERIAL AND METHOD

Cancer has been a notifiable disease in Norway since 1951. A detailed description of the cancer registration scheme has been given elsewhere (Pedersen and and Magnus, 1959). Frequent checks indicate that reporting of recognized cancer cases is very complete.

The present study is based on all new cases of primary lung cancer (Int. List No. 162) that according to reports were diagnosed in the total male population of Norway during the years 1953–56, altogether 724 cases of which 604 (83·4 per cent) had been examined histologically.

In 485 cases, or 80·3 per cent of those examined histologically, the classification by type was undertaken by Kreyberg. This was done in 1957, in connection with an investigation entirely unrelated to the present one (Host, 1960).

All pathological institutes in Norway very willingly contributed to the study by submitting unstained slides or paraffin blocks of nearly all their lung cancer cases. The pathological material of some of the cases had unfortunately been discarded, and for not a few others the material still available was considered inadequate for histological typing.

In the 119 cases that were not classified by Kreyberg the diagnoses originally made routinely by other pathologists have been used for the present study. A relatively large proportion of these cases was classified as " carcinoma ", with no further specification as to type, or as " undifferentiated carcinoma ".

On the basis of information available on case abstract cards in the Cancer Registry this lung cancer material was subdivided according to how the pathological specimen used for histological typing had been obtained, and the resulting distributions by type were studied. In the majority of cases material for histological examination had been obtained in more than one way. For example, in a large number of cases a specimen was first obtained by bronchoscopy, later lung tissue was removed by radical surgery, a third biopsy resulted when metastases developed, and finally material from a post-mortem examination became available. The successive specimens may have been examined in different laboratories. No attempt has been made to collect and re-examine all this pathological material. As a rule, only one specimen from each case has been re-examined, and whenever possible material from the primary tumour has been used. As a result, 551 cases (91·2 per cent) have been classified on the basis of examination of the primary tumour, while in the remaining 53 cases material from metastases only was available for typing.

With this procedure the information that can be gained from the lung cancer material is obviously more restricted than would have been the case had all successive specimens from each individual patient been re-examined and classified independently.

Findings

Table I shows the percentage distribution in the total material and in various subgroups. The subgroups are defined as follows :

B. *Small biopsy only* : Typing based on material obtained by bronchoscopy, supra clavicular node biopsy, exploratory thoracotomy, craniotomy, or other exploratory surgery. No autopsy.

C. *Biopsy from metastases only* : As above, except that only metastatic material was obtained by the biopsy. No autopsy.

D. *Material from radical surgery* : Typing based on material from lobectomy or pulmectomy. Autopsy may have been performed but has in no case had any influence on typing.

E. *Autopsy only* : Typing based exclusively on autopsy material. No biopsy performed ante mortem.

F. *All autopsy cases* : Autopsy performed in all cases but the material actually used for typing may have been obtained prior to death.

The definition of the various subgroups, and the allocation of cases to them, was influenced by the typing procedure described above. Note that all cases in

TABLE I.—*Primary Lung Cancer Among Norwegian Males, 1953–65. Percentage Distribution by Histological Type in Total Material and in Various Samples Selected on the Basis of How Material for typing had Been Obtained*

	Group I				Malignant tumour, other and unspecified type	Ratio, Group I/ Group II
	Epidermoid	Oat	Group II	Carcinoma, other and unspecified		
A. Total material (604 cases)	37·4	20·9	24·8	12·4	4·5	2·3
B. Small biopsy only (164 cases)	32·3	23·2	20·7	17·7	6·1	2·7
C. Biopsy from metastasis only (53 cases)	13·2	26·4	32·1	15·1	13·2	1·2
D. Material from radical surgery (180 cases)	61·1	8·9	18·3	7·8	3·9	3·8
E. Autopsy only (140 cases)	18·6	23·6	40·0	13·6	4·3	1·1
F. All autopsy cases (310)	31·0	26·1	28·1	11·3	3·5	2·0

Note.—Carcinoma, other and unspecified, includes "Carcinoma", not specified as to type, and "undifferentiated carcinoma".
Malignant tumour, other and unspecified type, includes "Mesothelioma", and a small number of unclassified malignant tumours.

subgroup *C* are included in *B*, all cases in *E* are included in *F*, and 54 cases in *D* are included in *F*. Four cases, none of them autopsied, are not included in any of the subgroups as the nature of the biopsy material could not be established with certainty.

The difference in type distribution between some of the subgroups are striking. For example, while the relative frequency of epidermoid carcinoma is 37·4 per cent in the total material, this type accounts for 61·1 per cent in the "Radical surgery" group, 18·6 per cent in the "Autopsy only" group, and merely 13·2 per cent in the "Metastases only" group. Oat-cell carcinoma ranges between 26·1 per cent in "All autopsies" and 8·9 per cent in "Radical surgery". The proportion of Group II tumours, on the other hand, varies less, 40·0 per cent and 18·3 per cent being the highest and lowest figures, in "Autopsy only" and "Radical surgery" respectively.

As one would expect, the relative frequency of "Carcinoma, other and unspecified" is particularly high in the "Small biopsy only" and in the "Metastases only", where typing often had to be based on scanty material obtained by bronchoscopy, pleura biopsy, or supra clavicular node biopsy.

The examination of the ratio Group I/Group II is to some extent disturbed by the high proportion of tumours of unspecified type in some of the subgroups.

Disregarding the unspecified tumours, the ratio is found to range between 3·8 in the " Radical surgery " group and 1·1 in the " Autopsy only " group. In the total material the ratio is 2·3.

DISCUSSION

The observed variation between the subgroups of the lung cancer material is statistically highly significant, which means that it is most unlikely to have arisen by chance. There are several possible explanations for the variation :

1. Table II, giving age-specific incidence rates, indicates a difference in age pattern of Group I and Group II tumours in the total lung cancer material. Samples of this material will consequently tend to contain varying proportions

TABLE II.—*Primary Lung Cancer Among Norwegian Males, 1953–56. Age Specific Indidence Rates per 100,000 Per Year of the Male Population, and Ratio Group I/Group II, by Age*

Type	Age groups					
	30–39	40–49	50–59	60–69	70–79	80 and over
Epidermoid } (Group I)	—	2·2	13·4	15·4	7·7	1·0
Oat } (Group I)	0·3	2·2	7·0	7·9	1·7	1·9
Group II	1·4	1·5	6·3	9·9	5·9	5·9
Carcinoma, other and unspecified	—	0·9	3·0	5·8	4·5	1·0
Malignant tumour, other and unspecified	0·2	0·3	1·2	2·1	0·7	—
All histologically examined cases	1·8	7·1	30·9	41·2	20·5	9·8
No histological examination	0·2	0·5	4·0	8·8	9·0	12·7
Total material, with and without histological examination	2·0	7·6	34·9	50·0	29·5	22·5
Ratio, Group I/Group II	0·2	2·9	3·2	2·4	1·6	0·5

of the two groups of tumours if for some reason the samples differ in age composition. Actually, as might be expected, the subgroups shown in Table I do differ somewhat in age composition, the greatest difference being between the two subgroups which also show the most marked dissimilarity in the distribution of histological types, namely the " Radical surgery " group and the " Autopsy only " group.

However, after age adjustment the variation in ratios Group I/Group II remains essentially unchanged. For the two subgroups mentioned the adjusted ratios are found to be 3·6 and 1·1 respectively, as contrasted with the unadjusted ratios of 3·8 and 1·1. Practically all of the variation thus remains unexplained.

2. The variation, or part of it, could conceivably arise from differences in the clinical characteristics associated with the various histological types of tumours. Thus, if tumours of a certain histological type are generally associated with a comparatively benign clinical course (slow local growth of tumour, late and limited metastasizing, long survival), then it seems justified to assume (*a*) that the typing of such tumours will rarely have to be based only on material from metastases, (*b*) that a high proportion of the cases with such tumours will undergo radical surgery, and (*c*) that relatively few of such tumours will be typed on the basis of autopsy material. The reasons for the last assumption are as follows : Firstly, a relatively large proportion of these cases will have died without recurrence of the malignant disease, thus denying the post mortem pathologist material for typing. Secondly, if—as in the present study—the material is a recent one so

that not all of the cases are dead at the time of analysis, then a relatively large proportion of those with the more benign varieties of tumours will still be slive, thus avoiding, so far, being included in a post mortem series.

Conversely, if tumours of a certain histological type are generally associated with a very malignant clinical course (rapid growth of tumour, rapid and widespread dissemination, short survival), then one may assume (*a*) that the typing of such tumours will relatively often be based on material from metastases (supra clavicular nodes, pleura, brain), (*b*) that a relatively small proportion of the cases with such tumours will undergo radical surgery, and (*c*) that a relatively high proportion will be typed on the basis of autopsy material. There will be relatively few survivors at the time the analysis is undertaken, which means that a high proportion of the cases have had the chance of appearing in a post mortem series, and in the great majority of those who have died tumour tissue will be demonstrable at the time of death.

Because of the early dissemination, which may be evident at the first examination, the very malignant type may more often than the moderately malignant type give rise to difficulties in determining the site of the primary tumour. There may accordingly be a greater tendency to request a post mortem examination in such cases.

Differences of the kind described, but of varying degree, do exist between some of the histological types in lung cancer. For example, compared with some of the other types epidermoid carcinoma may be characterized as moderately malignant, signalling a good chance of resectability and some hope of several years' survival. Among the 226 cases of epidermoid carcinoma in the present material, which as previously emphasized is a National material, 110 (48·7 per cent) were radically operated on. At the time of analysis—4 to 7 years after the diagnosis was made—30 of these cases were still alive and thus prevented so far from appearing in an autopsy series. While epidermoid carcinomas constitute 37·4 per cent of the total histologically examined material, 68·2 per cent of those still alive had tumours of this type.

In contrast, among the 126 cases with oat-cell carcinoma only 16 (12·7 per cent) were subjected to radical surgery. None of the patients with this tumour type are among the survivors. The highly malignant character of the oat-cell carcinoma is well recognized by the clinicians, many of whom regard a biopsy diagnosis of this kind as contra-indicating attempts at radical surgery, even when the tumour appears to be localized and, from a purely technical point of view, operable.

The regional distribution of epidermoid and oat-cell carcinoma in Norway is apparently very similar. Due to the more protracted course in epidermoid carcinoma, mostly allowing sufficient time for the diagnosis to be established while the tumour is still localized, a much high proportion of the cases with this tumour type that are diagnosed in the rural areas and smaller towns of Norway are considered suited for transference to the thoracic units in the Capital for treatment. Among 128 such cases from more or less remote area, 90 (70·3 per cent) were sent on to the University Hospitals in Olso, and among those so referred 57 (63·3 per cent) underwent radical surgery. Those who survived surgery and the majority of those not operated on returned to their local regions to die eventually in their homes or in the local hospitals where autopsy is rarely performed. Among 88 who died in the local region only 15 (17 per cent) were autopsied, while among

22 in this group who died during the first or subsequent admissions to the centres in Oslo 18 were autopsied. Eighteen patients are still alive.

In striking contrast to this picture, among 60 cases of oat-cell carcinoma diagnosed in rural areas and smaller towns, not more than 27 (45 per cent) were considered candidates for the thoracic centres. Only 3 were radically operated on. It is notable that among the 50 patients who died in their local region, as many as 10 (38 per cent) were autopsied. This is clearly higher than the figure for the corresponding epidermoid group, and very much higher than the National autopsy rate for all cancer cases (excluding skin cancer) dying outside the Capital (less than 10 per cent). This difference probably reflects the greater diagnostic difficulties, as already pointed out, in cases of oat-cell carcinoma, due to the early and distant spread in many of these cases. Apparently, in a relatively large proportion of the cases with this tumour type the classification of the case as primary lung cancer has remained uncertain until, or even after, the autopsy had been completed. The significance of this will be considered later.

Although the Group II tumours comprise some histological types that are often associated with a fairly benign clinical course (carcinoids, mucous gland tumours), the adenocarcinomas constitute by far the largest component of the Group—in the present material 107 out of 150 cases (71·3 per cent). According to Bignall (1958) the adenocarcinoma is generally a highly malignant type in lung cancer, and this is well born out by the Norwegian material. Among the 107 cases, 12 first reported with cerebral symptoms, due to brain metastases, and 5 underwent craniotomy. Radical resection of the lung tumour was carried out in 17 (15·9 per cent) of the cases with adenocarcinoma, but in 13 (30·2 per cent) of the other Group II tumours. Among 8 still surviving at the time of analysis only 1 had an adenocarcinoma, the other survivors being distributed as follows: 2 survivors among 8 with bronchiolo-alveolar carcinoma, 4 survivors among 31 with carcinoids, and 1 survivor among 4 with mucous gland tumours.

As in the preceding description of the clinical characteristics associated with epidermoid and oat-cell carcinoma, the patients from rural area and smaller towns will be used for a similar evaluation of the Group II tumours. Only rarely are patients from these areas admitted directly to the thoracic centres in the Capital. As a rule, they are kept under observation by the local hospital, or sometimes in a tuberculosis sanatorium, until the nature of the disease becomes sufficiently clear. At that time, some of the cases, predominantly the ones that are considered possible candidates for radical treatment, are transferred to the specialized centres. The proportion of cases thus transferred, and their fate, provide some interesting details for assessment of the behaviour of the disease. The influence of differences in age distribution is negligible in this context. Among 91 cases with Group II tumours diagnosed in rural areas and smaller towns, 51 (56 per cent) were sent to the centres in the Capital. For those with adenocarcinomas the percentage was 45, for the remainder 82. It is notable that among the 29 cases referred with an adenocarcinoma, not less than 9 were actually admitted to neurosurgical units with a diagnosis of brain tumour. Lung resection was carried out in 9 of the 29 patients with adenocarcinoma, and in 11 of the 22 patients with other tumour types included in Group II. Finally, of 72 cases with Group II tumours who died back in their home district (local hospital, private home) 28 (38·9 per cent) were autopsied—again a remarkably high figure which probably reflects the uncertainty of the clinical diagnosis in many of these cases.

More recent data kindly made available by Kreyberg (1961, personal communication) throw additional light on the characteristics of the various tumour types. In a series of 32 brain tumours representing metastases from primary lung tumours in males, only 4 were epidermoid, while 6 were oat-cell carcinomas, 20 adenocarcinomas, and 2 undifferentiated carcinomas. Independent typing of the brain metastases and the primary tumours in the lung gave identical results. This distribution is strikingly out of proportion with the recorded incidence of the various histological types in the population.

The data that have been presented indicate that there are marked differences between the histological types as regards the clinical manifestations accompanying them. It appears that in this respect the dividing line is not between Group I and Group II tumours but rather between, on the one hand, the highly malignant oat-cell carcinomas and adenocarcinomas and, on the other hand, more moderately malignant varieties dominated by the epidermoid carcinomas but including also some Group II tumours of minor quantitative significance. The differences in clinical behaviour are clearly such that they would tend to produce exactly the kind of variation displayed in Table I.

3. The site of origin of the primary tumour in the lung may have considerable influence on symptoms, diagnosis, treatment (resectability), and course of the disease (Bignall, 1958). Other things being equal, tumours developing in the central part of the lung seem to give rise to symptoms earlier than those originating in the peripheral part. While the former are, by definition, within the range of the bronchoscope, the latter are not—or not until considerable extension has occurred. To obtain a specimen from the primary tumour when it is situated peripherally generally requires thoracotomy or autopsy. On the other hand, the peripheral tumour is probably more readily recognized on the roentgenogram and is relatively often detected incidentally by mass roentgenographic surveys (Høst, 1960).

Tumours arising in different parts of the lung may have different anatomical relationships to lymphatics and blood vessels (Hinson, 1958). For that reason even if they do not differ in degree of malignancy, they may conceivably differ markedly as regards the time at which they metastasize and in their mode of spread.

Site of the tumour is therefore clearly relevant to our discussion. Unfortunately the present material contains only insufficient details regarding site, but a number of studies agree in showing that while the majority of epidermoid and oat-cell carcinomas originate centrally, adenocarcinomas and bronchiolo-alveolar tumours are predominantly situated peripherally (Hinson, 1958). As far as the epidermoid and oat-cell carcinomas are concerned there is apparently no difference in site that could explain the marked dissimilarities exhibited by the figures for these two types in Table I. However, between epidermoid and adenocarcinomas and, in general, between Group I and Group II tumours a difference in site distribution exists which is very likely to influence the frequency with which the various histological types turn up in the subgroups shown in Table I. But neither nature nor extent of this influence can be assessed in the available material.

4. The various pathological materials that are represented by the subgroups in Table I do not provide equally good opportunities for typing. In particular, the material in the "Small biopsy" and "Metastasis only" groups may quite often have been unrepresentative or the specimen actually too small for complete typing, and for these reasons the distributions shown may differ from the distribu-

tions that would have been obtained had more ample material from these cases been available for typing. The high proportion of " Carcinoma, other and unspecified " (the majority being unspecified) and " Malignant tumour, other and unspecified types " in the two subgroups mentioned, could clearly be a result of insufficient biopsy material.

The general question, whether there tends to be a systematic difference in classification when typing is carried out in various kinds of material from the same cases, could easily have been answered had all successive specimens from those cases where more than one specimen was obtained, been reviewed and typed independently.

5. As previously mentioned, 485 (80·3 per cent) of all cases in the present material have been typed by Kreyberg. The remainder have been typed by other pathologists in their routine diagnostic work. If now there is a systematic difference between Kreyberg and the other pathologists involved in regard to typing of lung cancer, and if the proportion of cases that have been typed respectively by Kreyberg and other pathologists differs between the subgroups in Table I, then variation in the histological type distribution would clearly result.

A difference between subgroups as regards their distribution among pathologists actually exists. A relatively large proportion of the autopsy series has not been typed by Kreyberg. Thus, among the cases in the " Autopsy only " group only 66·4 per cent have been typed by him, as compared with 80·3 per cent in the total material, and 88·9 per cent in the " Radical surgery " group.

There is also, in the present material, a notable difference between Kreyberg and the other Norwegian pathologists involved as regards typing. It must be remembered, however, that the classifications furnished by " other pathologists " are unrevised, that they have been made as part of the daily routine, and that the motivation for giving highly specified diagnoses may not always have been very strong.

In order to get some idea of the extent and nature of the " disagreement " between the pathologists involved in typing a cross-tabulation was made of 276 cases all of which had been typed independently by Kreyberg and " other pathologists ". Complete agreement of type was found in 160 cases (58 per cent). By far the greatest discrepancy was in the proportion of cases classified as " Carcinoma, other and unspecified ", or as " Malignant tumour, other and unspecified types ". While 28 cases (10 per cent) were thus classified by Kreyberg, for " other pathologists " the figure was 105 (38 per cent). Notable " disagreement " was also found in the proportion of cases classified as Group II, the figures being 86 cases (31·2 per cent) for Kreyberg and 35 cases (12·7 per cent) for " other pathologists ". There was less " disagreement " regarding epidermoid and oat-cell carcinomas, but it is noted that cases classified as malignant adenoma by Kreyberg tended to be classified as epidermoid or oat-cell carcinomas by " other pathologists ".

The only comparison in Table I that is markedly affected by these dissimilarities in classification is the one between the " Radical surgery " and " Autopsy only " groups. Among the 140 cases in the latter group, 93 were typed by Kreyberg with the following result (percentages) :

Epidermoid	Oat	Group II	Carcinoma, other and unspecified	Malign tumour, other and unspecified	Ratio GI/GII
18·3	20·4	51·6	6·5	3·2	0·8

Assuming that there is no real difference between the 66·4 per cent of this group that have been classified by Kreyberg and the 33·6 per cent that have been classified by " other pathologists " only, this is approximately the distribution to be expected in the " Autopsy only " group had all cases been typed by Kreyberg. The essential changes, compared with the distribution in Table I, are a material increase in the proportion of Group II tumours, and a reduction in the proportion of unspecified tumours. The ratio Group I/Group II is reduced to 0·8.

It seems justified to conclude that had the proportion typed by Kreyberg been as high for the autopsy series as for the other subgroups of the material, then the variation in type distribution would have been no less than that displayed in Table I. Probably the variation would have been greater, in particular between the " Radical surgery " and " Autopsy only " groups.

Although in the present study typing conditions were very dissimilar for Kreyberg and " other pathologists " the findings just described may serve as a reminder of the problem of differences between observers in studies of this kind.

6. Blind typing has not been possible in this study. Usually the character of the pathological material has been known to the pathologist. When observer bias is nevertheless assumed to be negligible as a cause of variation it is mainly for the following reasons: Firstly, Kreyberg, who has undertaken by far the greater part of the typing, has repeatedly demonstrated that his classification, under varying conditions, including blind trials, is highly consistent (Doll, Hill and Kreyberg, 1957; Kreyberg and Saxén, 1961). Secondly, as pointed out before, the typing on which this analysis is based was actually undertaken in connection with a study entirely unrelated to the present one.

CONCLUSIONS

An attempt has been made to discuss some factors that might explain the marked dissimilarity in relative frequency of the various histological types in different subgroups of a National lung cancer material, the subgroups being selected on the basis of how pathological material for typing has been obtained. It seems that differences in clinical characteristics associated with the various histological types may easily account for the greater part of the variation, if not for all of it. The relative role played by site of the tumour (central or peripheral) and degree of malignancy *per se* in determining these clinical characteristics cannot be assessed in the present material. Although the other factors that have been discussed are definitely of interest from a methodological point of view, their influence is probably very modest in the present material.

A final word must be added regarding the nature of the present material. It is unselected in the sense that it includes all histologically confirmed cases of primary lung cancer diagnosed among the male population of Norway during a defined period of time. However, among the total number of cases that were classified as primary lung cancer during the period, 16·6 per cent had not been confirmed histologically. This is a relatively small proportion, but Table II shows that their age pattern—a steady increase with age—differs markedly from that of all histologically confirmed cases. It is indeed possible that this undetermined group—or that part of it which really is primary lung cancer—contains the varying histological types in proportions different from those observed in the histologically examined group.

Furthermore, as previously emphasized, the various histological types seem to present diagnostic problems of varying degree. In highly malignant cases, frequently presenting with widespread metastases on first examination, it may be particularly difficult to demonstrate in which organ the primary tumour is situated. It is entirely possible that among the total cases of lung cancer arising in a population during a given period of time, the proportion that is correctly recognized is highly dependent upon clinical characteristics and, consequently, upon histological type.

The essence of these remarks is that our "National material" constitutes merely a sample, more or less biased, of the total lung cancer cases arising in the population during the defined period. Any change in diagnostic standards affecting the recognition or rate of histological examination of lung cancer cases is likely to influence the recorded relative frequency of the various histological types. It is important to bear this in mind in comparisons involving different regions or different time periods, as both the recognition of lung cancer cases and the frequency of histological examination of recognized cases are likely to vary considerably with time and place.

The author is very grateful to Professor Kreyberg for his advice and criticism during the preparation of this paper.

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METASTASIZING BRONCHIAL ADENOMA WITH ASSOCIATED CARCINOID SYNDROME

M. I. SACKS* AND A. H. TIMME

From the Department of Pathology, University of Cape Town Medical School, Observatory, Cape, South Africa

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IT is now generally accepted that the histological similarity between the carcinoid type of bronchial adenoma and the argentaffine tumour of the gastro-intestinal tract reflects a close relationship between these two neoplasms. Thus in the past few years several cases have been reported in which metastasizing bronchial adenomas have been associated with the carcinoid syndrome but there are few detailed autopsy studies of such cases.

In a report of 11 clinical cases of the syndrome, Krikler, Lackner and Sealy (1958) included one in which the primary tumour was thought on clinical and radiological grounds to be situated in the bronchus. The purpose of the present paper is to record the detailed pathological findings in this case as the diagnosis has now been confirmed at autopsy. The results of histochemical and biochemical studies will also be recorded.

Clinical History

The patient, a European (white) female, aged 49 years, was first seen in March, 1957, complaining of flushing of the face, painless watery diarrhoea and nocturnal wheezing of three months' duration. She showed a reddish-blue complexion. The blood pressure was 170/100 mm.Hg. but further clinical examination of the cardiovascular and respiratory systems disclosed no abnormality. The liver was enlarged to 6 cm. below the costal margin, firm, nodular and slightly tender. A warm red nodule, 2 cm. in diameter, was present in the skin of the right shoulder. Blood examination: haemoglobin 15·5 g. per cent; E.S.R. 10 mm. in 1 hour (Westergren); W.B.C. count 8,000 per cu. mm. X-ray of the chest showed a nodular mass in the hilar region. Urinary 5-hydroxy-indole acetic acid (5-H.I.A.A.) 210 mg. per 24 hr.

Liver biopsy showed tumour tissue with a papillary structure and consisting of small uniform cells with non-granular cytoplasm arranged around blood vessels. Both the Masson's stain and the diazo reaction were negative. The shoulder nodule contained tissue similar to that seen in the liver though without the papillary structure. Much of it was necrotic and surrounded by dense collagenous tissue. A 12-day course of deep X-ray therapy to the pulmonary lesion was given in June, 1957, (dose 2,250 r) with no symptomatic relief or radiological response. In July, 1957, a tumour dose of 2,700 r to the whole of the liver over a 4-week period gave prompt relief in that the diarrhoea and bronchospasm ceased and her complexion became normal, but the liver did not decrease significantly in size. The

*Present address: c/o The Department of Pathology, Hadassah University, Jerusalem, Israel.

patient experienced only very occasional diarrhoea and mild flushing attacks during the following year.

In August, 1958, right-sided sciatic pain was relieved by a 14-day course of deep X-ray therapy to the spine (dose 3,000 r). At this time radiological examination showed probable osteosclerotic metastases in the second and fourth lumbar vertebrae but there was little change in the size of the left hilar mass.

The patient felt relatively well for 6 months but in June, 1959, her earlier symptoms reappeared. The urinary 5-H.I.A.A. excretion was 190 mg. per 24 hr. In August, 1959, 15 g. Thio-TEPA was given by daily intravenous injection. Two weeks later the liver was smaller than before and non-tender but there was no significant decrease in the 5-H.I.A.A. excretion. Hepatic pain later returned and although there were no pulmonary symptoms the hilar shadow had enlarged. A course of CB 1348 was without response.

On 18 July, 1960, she was admitted with severe diarrhoea (14 yellow watery stools daily) and dyspnoea on effort. Blood pressure was 140/85. No cardiac murmurs were heard. Pulse rate was 94 per min. Haemoglobin 12 g. per cent; W.B.C. count 11,000 per cu. mm.; platelet count 413,000 per cu. mm.; serum bilirubin 0·4 mg. per cent; serum albumin 3·2 g. per cent; serum globulin 2·0 g. per cent; serum alkaline phosphatase 5·1 Bodansky units; thymol turbidity 1; zinc turbidity 11. The urinary 5-H.I.A.A. ranged from 67 to 141 mg. per 24 hours and the urinary output from 840 to 1880 ml. per 24 hr. Her temperature was 101° F. A 9-day course of deep X-ray therapy to the liver was given but she became confused and disorientated and died on 13 August, 1960.

Autopsy Findings

The autopsy was performed 34 hr. after death. The subject was very thin and the little subcutaneous fat remaining had a bright yellow colour. The skin over the lower lumbar spine was pigmented and thickened presumably as a result of the irradiation. There was no oedema or jaundice.

Dense adhesions obliterated the anterior portion of the left pleural sac but posteriorly there was about 100 ml. of slightly haemorrhagic fluid while a plaque of tumour tissue bound the left lower lobe to the diaphragm. Right-sided apical adhesions were also present.

The left lung weighed 340 g. and the right 660 g. A soft grey-white polypoid tumour measuring 1·2 × 1 × 1 cm. was situated in the left main bronchus 4 cm. beyond the tracheal bifurcation and just proximal to the point of division of the bronchus into its two main branches. The intrabronchial tumour was continuous through gaps in the cartilage with a much larger encapsulated mass 7 × 5 cm. which occupied the greater part of the lower lobe and extended to the pleural surface (Fig. 1). This portion of the tumour was firm and also greyish-white in colour. Near the hilum of the lung there were three further soft and haemorrhagic nodules demarcated from each other by fibrous trabeculae. The left inferior pulmonary vein was compressed and surrounded by the tumour but not thrombosed.

The right lung was moderately oedematous. The bronchi appeared normal. Two small rounded white tumour deposits, 0·8 and 1·2 cm. in diameter, were present, the smaller being intimately related to the adventitia of a medium-sized pulmonary artery. A left-sided paratracheal lymph node and two nodes in the left supraclavicular fossa were infiltrated by opaque white tumour.

The heart weighed 252 g. and showed no dilation of either ventricle nor were any thrombi found in any chamber. A few nodules of tumour were present on the inner aspect of the parietal pericardium adjacent to the mediastinal surface of the left lung. The endocardium of the right auricle just above the tricuspid valve was thickened and opaque. The tricuspid valve cusps were thickened and more opaque than normal, and there was slight thickening and shortening of the chordae tendineae. The cusps were not adherent to each other and the valve ring measured 9·5 cm. in circumference. A few small areas of endocardial thickening were seen over the trabecular muscles in the right ventricle. The pulmonary valve appeared normal, the valve ring measuring 7·0 cm. in circumference. The mitral valve was normal but ridge-like thickenings were seen on the aortic valve cusps along the lines of contact. The myocardium appeared normal. A moderate number of atheromatous plaques were present in the right coronary artery, but the left was virtually free.

No ascites was present but there were a few adhesions between the right lobe of the liver and the ascending colon. Multiple rounded tumour nodules, 0·5 to 2·0 cm. in diameter, projected from the outer surface of the liver (2138 g.), several of them being surrounded by a depressed zone. Along the anterior border of the right lobe was a deep depressed scar. A granularity of the external surface of the left lobe suggested the presence of a cirrhosis. On section multiple tumour deposits were found throughout both lobes, at least 20 being seen on the initial plane of section. The larger ones had undergone extensive haemorrhage and necrosis. The intervening hepatic tissue showed a Laennec type of cirrhosis. No thrombi were found in the portal or hepatic veins.

A rounded tumour deposit, 2·5 cm. in diameter, was found in the head of the pancreas immediately adjacent to the second part of the duodenum. The rest of the alimentary tract, including the appendix, showed no abnormality apart from a small gastric polyp.

The thyroid gland was enlarged. It contained several colloid-filled nodules measuring up to 2 cm. in diameter. The intervening thyroid tissue was firm in consistency.

The left femur contained numerous circumscribed tumour deposits while several of the lower thoracic and upper lumbar vertebrae were diffusely infiltrated by sclerotic opaque white tissue. The other long bones were not sectioned. A round tumour deposit was present in the left parietal bone of the skull and the bone surrounding the tumour appeared sclerosed.

No tumour deposits or any other lesions were found in any other organ.

Histology

The bronchial tumour showed the features of a carcinoid type of bronchial adenoma. The tumour was composed of small uniform cells which were mostly polygonal in shape but columnar when situated perivascularly. The cytoplasm was faintly eosinophilic and usually non-granular but occasional groups of tumour cells could be found in most sections which did contain granules. The nuclei were small and round or ovoid in shape and had a fine chromatin pattern but no nucleoli. The arrangement of the tumour cells varied according to the amount of stroma present and the vascularity. In some areas there were solid islands of varying sizes composed of closely applied tumour cells separated by broad bands of hyaline fibrous tissue. Elsewhere a complex ribbon-like arrangement of the cells was

seen (Fig. 2) and here the tumour showed a striking vascularity. The columnar cells were grouped around capillaries producing pseudo-rosettes but a few true acinar structures were also seen (Fig. 3). Areas of cystic degeneration containing faintly eosinophilic P.A.S. positive but mucicarmine negative material were plentiful in some sections. No mucicarmine positive material could be identified. Lying between the tumour cells were occasional calcified spherules two or three times the size of the cells. The intrabronchial portion of the tumour was ulcerated and contained abundant collagen in its most superficial portion. Blood vessels within the tumour showed a conspicuous thickening of their walls which had a pale hyaline structureless appearance devoid of recognisable nuclei but containing small rounded basophilic structures, the latter possibly representing nuclear debris (Fig. 4). No elastic fibres or smooth muscle were seen in these vessel walls but there were very fine collagen fibres. The tumour did not show much necrosis but in some areas autolytic changes were in evidence as the cells were widely separated from each other and the nuclei were pyknotic. Vessels similar to those seen in the tumour were also found in the uninvolved lung tissue.

The tumour deposits in the liver were similar to those seen in the lung but cystic degeneration was more prominent. Several deposits showed extensive central necrosis. The larger ones had a fairly broad fibrous capsule. The deep scar on the anterior surface of the liver consisted of broad bands of fibrous tissue in which were embedded small groups of residual tumour cells and many proliferating bile ducts. The portions of liver unaffected by tumour showed moderate or fairly severe fatty change and thin bands of fibrous tissue linking up thickened portal tracts.

The right auricular endocardium contained an increased number of elastic and collagen fibres. The tricuspid valve cusps were markedly thickened. Much of this was due to a myxomatous change but elsewhere there was also an increase in hyaline fibrous tissue. Very few elastic fibres were seen, and those present were aggregated towards the auricular surface. Near the free margin of the cusp a loose and myxomatous connective tissue containing delicate elastic fibres appeared to have been superimposed on its auricular surface (Fig. 5). The cusp contained no cellular infiltrate. Towards its base there was an increase in vascularity. The thickening of the chordae had resulted from a deposition of loose collagenous tissue around them. The pulmonary valve was slightly thickened and near its free margin was a focal nodularity on the concave surface which again was composed of loose collagenous tissue lying superficial to the elastic lamina of the cusp. The ridge-like thickening of the aortic valve was due to a similar lesion. The mitral valve, though macroscopically normal, showed changes of a similar nature with a plaque-like layer of collagenous tissue lying superficial to the elastic lamina of the cusp on its ventricular surface. Small perivascular foci of fibrosis were seen in the myocardium, and larger vessels in the subpericardial adipose tissue were surrounded by a cuff of pale fibrous tissue.

The thyroid showed a pronounced degree of fibrosis which replaced large areas of thyroid parenchyma leaving only a few small atrophic follicles lined by Askanazy cells. Elsewhere the follicles varied greatly in size and formed nodules separated by fibrous trabeculae. A lymphocytic infiltrate was present in the areas of most marked fibrosis. Plasma cells were inconspicuous.

The bony metastases were associated with an osteosclerotic reaction and some periosteal new-bone formation. The tumour mass in the pancreas had infiltrated

the outer layers of the muscle coat of the duodenum but the mucous membrane and submucosa were intact. Sections from the remaining organs showed no noteworthy changes.

Histochemical Investigations

The tissues examined included the liver and skin biopsies and those sections of the tumour obtained post mortem in which autolysis was minimal and the cytoplasmic granules best preserved. After the post mortem interval of 34 hours, the tissues were fixed in 10 per cent formol-saline. Unless otherwise specified, surgically removed appendices were used as controls.

Bodian's technique was used to demonstrate argyrophilia (Bodian, 1936). Sections of the lung tumour and hepatic metastases showed that about 40 per cent of the cells contained black cytoplasmic granules (Fig. 6). The Masson-Fontana argentaffin reaction and Schmorl's test were performed according to Pearse (1960) but were negative in both biopsy and post mortem sections. The diazo coupling reaction using Fast Red Salt B (Pearse, 1960; Lillie and Glenner, 1960) was negative in all sections examined. The granules within the tumour cells did not stain with P.A.S. and no lipoid was demonstrable in frozen sections stained with Sudan III. The method of Adams (1957) for tryptophan stained the tryptophan-containing zymogen granules of a surgically removed pancreas a greenish-blue colour but the carcinoid granules did not stain.

Fluorescence.—The sections were initially examined without prior removal of wax (Barter and Pearse, 1955) but better results were obtained after dewaxing the sections in xylol and mounting them in a non-fluorescent balsam. An intestinal carcinoid which gave positive diazo and argentaffin reactions served as a positive control. A smear preparation of 5-H.T. was also made and after exposing this to formalin vapour (Barter and Pearse, 1955) this gave a similar orange-yellow fluorescence to that of the intestinal carcinoid. Sections of a carcinoma of the cervix and of the stomach served as negative controls. Several sections of the bronchial carcinoid and metastases removed at post mortem did not fluoresce.

Biological assay.—Unlike a control solution of 5-H.T., an N/50 HCl extract of a portion of tumour tissue from our case did not cause contraction of a rat uterus or colon.

Biochemical Investigations

For these studies we are greatly indebted to Mr. E. J. Duncan and Professor J. E. Kench of the Department of Chemical Pathology.

The material studied consisted of unfixed tumour, lung and liver tissue obtained post mortem and immediately placed into a deep freeze. Specimens of tumour

EXPLANATION OF PLATES

FIG. 1.—The polypoid intrabroncial portion of tumour is seen to be continuous with a larger lobulated mass in the substance of the lung.

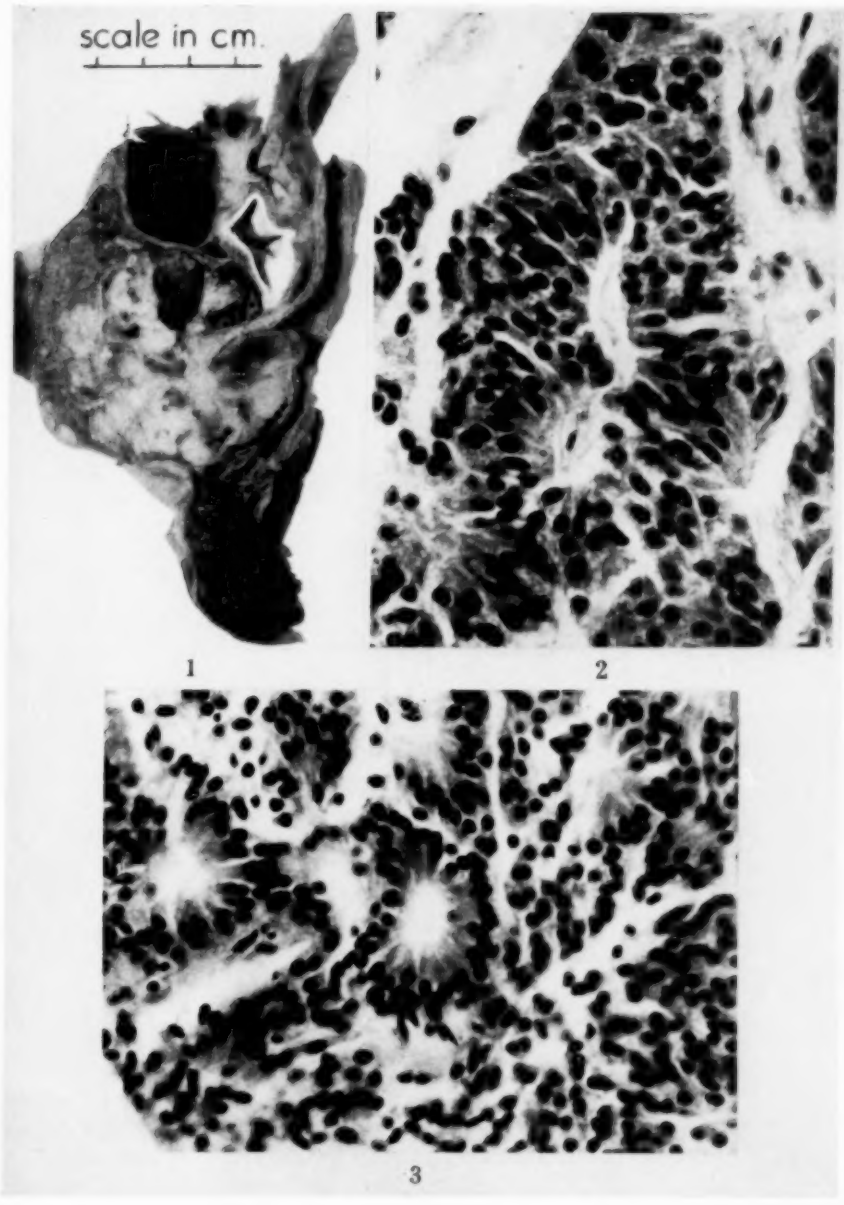
FIG. 2.—The tumour cells are arranged in anastomosing columns. ×100.

FIG. 3.—This shows the tumour forming small acinar spaces. ×100.

FIG. 4.—A blood vessel in the lung tumour. The basophilic structures thought to represent nuclear debris can be seen in the grossly thickened walls. ×90.

FIG. 5.—The tricuspid valve. Verhoeff's elastic stain. A loose connective tissue containing elastic fibres has been deposited on the auricular surface of the valve cusp. ×22.

FIG. 6.—This shows the argyrophilic cytoplasmic granules in the tumour cells. ×90.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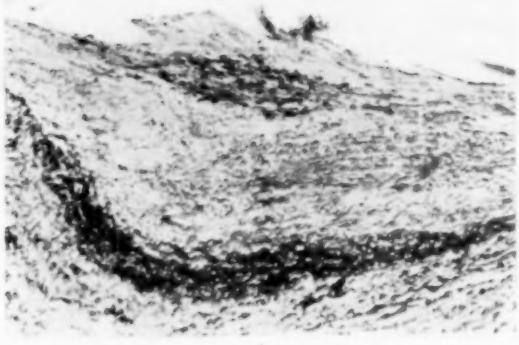

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and of uninvolved liver tissue from a case of gastric carcinoma were used as controls. The tissue was finely ground and extracted with acetone which was evaporated *in vacuo*, and the residue made up to a definite volume with water and defatted with light petroleum. The determination of 5-H.I.A.A. was carried out as described by Udenfriend, Titus and Weisbach (1955) and the figures obtained by this method include all 5-hydroxyindole compounds.

The following results were obtained :

I.—*Case of Carcinoid Syndrome*

Uninvolved lung	. .	0.68 mg. per cent
Carcinoid tumour in lung	.	2.29 mg. ,, ,,
Uninvolved liver tissue	.	0.53 mg. ,, ,,
Carcinoid tumour in liver	.	1.91 mg. ,, ,,

II.—*Control Case*

Carcinoma of stomach	.	1.54 mg. ,, ,,
Liver tissue	. . .	1.41 mg. ,, ,,

Extracts of the tumour were also investigated by paper chromatography using a 2-dimensional system—(i) Isopropanol-ammonia, (ii) Butanol-acetic acid. The spots were located by Ehrlich's Aldehyde Reagent.

(1) *Standard solution.*—5-H.T., 3-methylindole (skatole), 3-H.I.A.A., 5-H.I.A.A., tryptophan and urea were used and could be readily separated and identified.

(2) *Uninvolved liver tissue.*—Skatole, 5-H.I.A.A. and urea were present but no 5-H.T. was detected.

(3) *Tumour in Liver.*—5-H.I.A.A., urea, skatole and indole were present. No 5-H.T. could be detected. A red spot (? porphobilinogen) was also noted.

(4) *Uninvolved lung.*—Tryptophan, urea, skatole and indole and a faint trace trace of 5-H.I.A.A. were present.

(5) *Tumour in lung.*—Tryptophan, 5-H.I.A.A. and skatole but no 5-H.T. were present. A red spot (? porphobilinogen) was noted.

Recovery experiments were then performed in which 0·5 mg. of 5-H.T. was added to the tumour and extractions were done as before and investigated chromatographically. When the extract was made immediately after adding the 5-H.T. the presence of 5-H.T., 5-H.I.A.A., skatole, urea and ? porphobilinogen could be demonstrated, but when the extract was made after allowing the tumour and added 5-H.T. to stand at room temperature for 60 min., there was only a faint trace of 5-H.T. and skatole, but no 5-H.I.A.A. nor tryptophan. Similarly, two portions of tumour tissue, each weighing 2 g., were mixed with 0·5 mg. serotonin. One portion was allowed to stand at room temperature for 60 minutes and the other was extracted immediately. In the latter case the recovery was 0·202 mg. (theoretical yield 0·2375 mg. 5-H.I.A.A.) whereas if the extraction was delayed the yield was 0·006 mg.

As a control in the recovery experiments 0·5 mg. 5-H.T. was mixed with portions of the gastric carcinoma used in the earlier estimations and allowed to stand at room temperature before extraction. The theoretical concentration before

extraction was 0·2618 mg. per 2 g. of tumour and the true yield was 0·2162 mg. per 2 g. Chromatographic examination of acetone extracts of the gastric carcinoma showed spots of 5-H.I.A.A. and urea. When the tumour was mixed with 0·5 mg. of serotonin and extracted after an hr. at room temperature the spots of 5-H.T., 5-H.I.A.A. and urea were still present.

DISCUSSION

The purpose of our paper has been to record the autopsy findings in a patient with the clinical and biochemical findings of the carcinoid syndrome and with the primary tumour in the bronchus. The morbid anatomical and histological features of the lung tumour were typical of the carcinoid type of bronchial adenoma and careful examination of the gastro-intestinal tract at autopsy disclosed no primary intestinal carcinoid. The clinical and pathological features of previously reported cases of the carcinoid syndrome due to metastasising bronchial carcinoids have recently been reviewed (Williams and Azzopardi, 1960; Weiss and Ingram, 1961) and will not be discussed here.

Certain aspects of our case warrant special comment although their true significance may only emerge when further cases of this type have been studied.

With the exception of the non-specific argyrophilic reaction the histochemical tests in our case were negative *in* all sections examined. It is well recognised that the argentaffin reaction may be negative in tumours producing the syndrome (Thorson, 1958; Waldenström, Pernow and Silwer, 1956), including cases in which the tumour has been found to contain large amounts of 5-H.T. (Smith *et al.*, 1957; Thorson, 1958). Therefore it has been postulated that variable staining reactions may, in some cases, be partly related to different secretory activities of the cells rather than autolytic changes. It has not been clear, however, whether the tumours in the cases referred to includeed cells containing histologically recognisable granules in their cytoplasm. Williams and Azzopardi (1960) have recently suggested that the granules represented a storage product which retains the soluble 5-H.T. in the cytoplasm. It is consequently of considerable interest that in our case, in which the granularity of certain groups of cells was a prominent feature, the specific histochemical tests were negative. The non-reactivity of the cells is furthermore reflected in the failure to demonstrate 5-H.T. by biological and chemical methods. We would therefore suggest that any 5-H.T. originally present in the granules could have disappeared during the long post mortem interval without disturbing the structure of the granules, and thus accounting for their histochemical non-reactivity.

The biochemical studies failed to reveal any 5-H.T. in the tumour tissue, which did, however, contain a greater level of 5-hydroxy-indole compounds than was present in the adjacent univolved tissues. It was of interest that the level of 5-hydroxy-indole compounds was greater in the control specimens than in the tumour-free lung and liver from our case. The post mortem intervals were similar in the two cases. Possibly related to this finding is the apparent disappearance of 5-H.T. added to the carcinoid tumour *in vitro*, whereas this did not occur when 5-H.T. was added to the gastric carcinoma. It must remain conjectural whether or not this represents a true enzymatic destruction of the 5-H.T.

Of the four recorded autopsy cases in which the carcinoid syndrome was associated with a primary bronchial tumour only that of Weiss and Ingram (1961)

showed the typical cardiac abnormality. The tricuspid valve lesions in our case corresponded to those described in some cases by Thorson (1958) in that a loose collagenous tissue had been superimposed on a cusp which itself was myxomatous but elsewhere contained an increased amount of collagen. The pulmonary valve changes, consisting of a collagenous tissue lying superficial to the elastic lamina of a relatively normal cusp, are similar to those described by MacDonald and Robbins (1957) and to the superficial fibrous pad mentioned by Thorson (1958), and may represent an earlier lesion than that seen in the tricuspid valve. Although Smith and Campbell (1956) suggested that the valves on the left side of the heart may be involved more frequently than is generally recognised, the assessment of these alterations is complicated by the fact that these valves not infrequently show non-specific nodular thickenings in autopsies on adult patients, and the changes we have described may well be of this type.

The changes in the thyroid resembling Hashimoto's disease and the cirrhosis are of interest in view of the fibrogenic properties of serotonin. The literature contains very few references to the thyroid, and although fatty change of the liver and cirrhosis have rarely been recorded (MacDonald, 1956; Zarafonetis, Lorber and Hanson, 1958), their significance is uncertain. A further complicating factor in our case is that the liver had been irradiated, and although the deep scar along the anterior border of the liver appeared to be secondary to spontaneous or irradiation induced necrosis of tumour, it would be difficult to account for the more diffuse fibrosis elsewhere on this basis.

The hyaline thickening of the pulmonary arteries in our case is worthy of mention as similar changes were described by Hand, McCormick and Lumb (1958). Although noted in both lungs, they were more prominent on the left side. An irradiation effect therefore had to be considered, but there were no other signs of a radiation pneumonitis. Furthermore, we have observed similar though less pronounced hyaline thickenings of small pulmonary arteries in autopsies on middle-aged subjects, and the significance of these changes must remain open to question. Bone metastases have only infrequently been recorded in metastasising intestinal or bronchial carcinoids. Toomey and Felson (1960) drew attention to the usual osteosclerotic nature of the bony secondaries and this feature was also seen in our case.

Radiation therapy of carcinoid tumours has received scant attention but Pearson and Fitzgerald (1949) refer to isolated reports suggesting that the tumours were radiosensitive. Irradiation of the liver resulted in temporary symptomatic relief in our patient and similar treatment to the spine relieved her sciatica.

SUMMARY

The autopsy findings have been recorded in a patient who presented the clinical and biochemical features of the carcinoid syndrome in association with a metastasising bronchial adenoma.

Failure to demonstrate 5-hydroxytryptamine in the tumour or its metastases by histochemical methods was reflected in an absence of chromatographically or biologically detectable amounts of the substance.

Attention has been drawn to the presence of cirrhosis of the liver and to changes resembling those of Hashimoto's disease in the thyroid gland.

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RETICULUM CELL SARCOMA IN SIBLINGS
(Report of One Family)

FLORA SMITH

From the Department of Pathology, Auckland Hospital, New Zealand

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In the more common types of cancer the presence of several cases of cancer in the same family does not necessarily indicate a familial inherited predisposition. Many genes are possibly involved in determining susceptibility to the common cancers (Koller, 1959). In the less common forms of cancer and in those occurring in childhood the part that heredity plays is not known with any certainty.

In the two cases to be described two brothers each developed a reticulum cell sarcoma in the third year of life and died after a few months. The first child was dying of the disease when the second child was born. The tumour had a similar distribution in both cases affecting the terminal ileum, caecum, ascending colon and lymph nodes. The diagnosis was established during life by biopsy in both cases.

REPORT OF CASES

Family history

Both parents (Mr. and Mrs. B.) were of European descent and were not related in any way. They were healthy and intelligent. Their respective parents were still alive. Some of the grandparents were still alive and those dead reached advanced years. Mrs. B. had four half brothers and one half sister and there was nothing significant in their family histories. Those that married have had healthy children. Mr. B. lost two sisters in infancy at the age of three weeks and three months. These deaths were thought to be due to congenital heart disease and inflammation of the stomach respectively. Mr. B. has two brothers and two sisters alive. Two brothers and one sister are married and they have healthy children.

Mrs. B. was aged 28 years when her elder son was born and Mr. B. was then aged 32 years. Mrs. B. gave no history of any miscarriages. There were only two children in this family.

The blood grouping of Mrs. B. is group BD negative, Genotype : ccddee D^u negative.

The blood grouping of Mr. B. is group AD negative, Genotype : ccddee D^u negative.

Case 1

L.B. was born on June 12, 1953. He was first admitted to Auckland hospital in September 1955 for investigation of irritability, anorexia, loss of weight and anaemia of six weeks' duration. On examination a mass was felt in the right iliac fossa and at laparotomy on September 19, 1955, an abscess was found which

was considered to be appendicular in origin. Postoperatively he developed a faecal fistula and spent several weeks in hospital before this cleared. The fistula recurred and he was readmitted on November 20, 1955, two weeks after discharge. On examination a mass was still palpable in the right iliac fossa and the fistula was discharging faecal material. On December 12, 1955, a further laparotomy was performed and a large abscess cavity was found in the right iliac fossa with bowel (not certain whether large or small bowel) adherent to it. A piece of the wall of this cavity on histological examination showed infiltration with an undifferentiated malignant neoplasm (Fig. 1). The leucocytes were never higher than 22,000 per cu. mm. with neutrophils predominating. The haemoglobin fell as low as 8·7 g. This patient belonged to group B and was D negative.

There was a steady deterioration in his condition until death on February 29, 1956.

Post mortem findings.—The autopsy was performed 9 hr. after death. The child was very emaciated. An infected area was present in the lower central abdominal wall with two fistulous openings present communicating with the terminal ileum. A full autopsy was carried out and the main interest was in the alimentary system and lymph nodes. A cavity containing watery faecal material was present in the right iliac fossa bounded ventrally and laterally by the abdominal wall and on its other aspects by caecum and numerous loops of small bowel. Several loops of ileum, the caecum and adjacent ascending colon were bound together by a mass of firm white tumour tissue which infiltrated the walls but without apparent ulceration of the mucosa. There was considerable distortion and the ileo-caecal valve could not be identified. The omentum was also adherent to the mass and infiltrated by tumour, this extending through it to infiltrate the inferior border of the liver. A large mass approximately 8 × 5 × 5 cm. was present at the base of the mesentery. Further tumour masses involving lymph nodes extended down the aortic group of nodes, into the hypogastric region, and along both external iliac vessels and into both inguinal regions. The latter nodes were of walnut size. The sigmoid colon was also adherent to the mass in the right iliac fossa and its wall invaded by tumour. The spleen weighed 38 g. and appeared normal when cut. The other organs appeared normal.

Histological examination.—Malignant tumour was seen replacing the lymphoid tissue in the inguinal, (Fig. 2 and 3) iliac and retro-peritoneal lymph nodes. It

EXPLANATION OF PLATES

FIG. 1.—Case 1. Wall of vermiform appendix showing infiltration by malignant tumour. (H. and E. × 330)

FIG. 2.—Case 1. Inguinal lymph node showing replacement by a reticulum cell sarcoma. (H. and E. × 330).

FIG. 3.—Case 1. Inguinal lymph node showing reticulum fibres in a reticulum cell sarcoma. (Gordon and Sweets Reticulum Impregnation × 330).

FIG. 4.—Case 1. Ascending colon showing infiltration of muscular layers by a reticulum cell sarcoma. (H. and E. × 85).

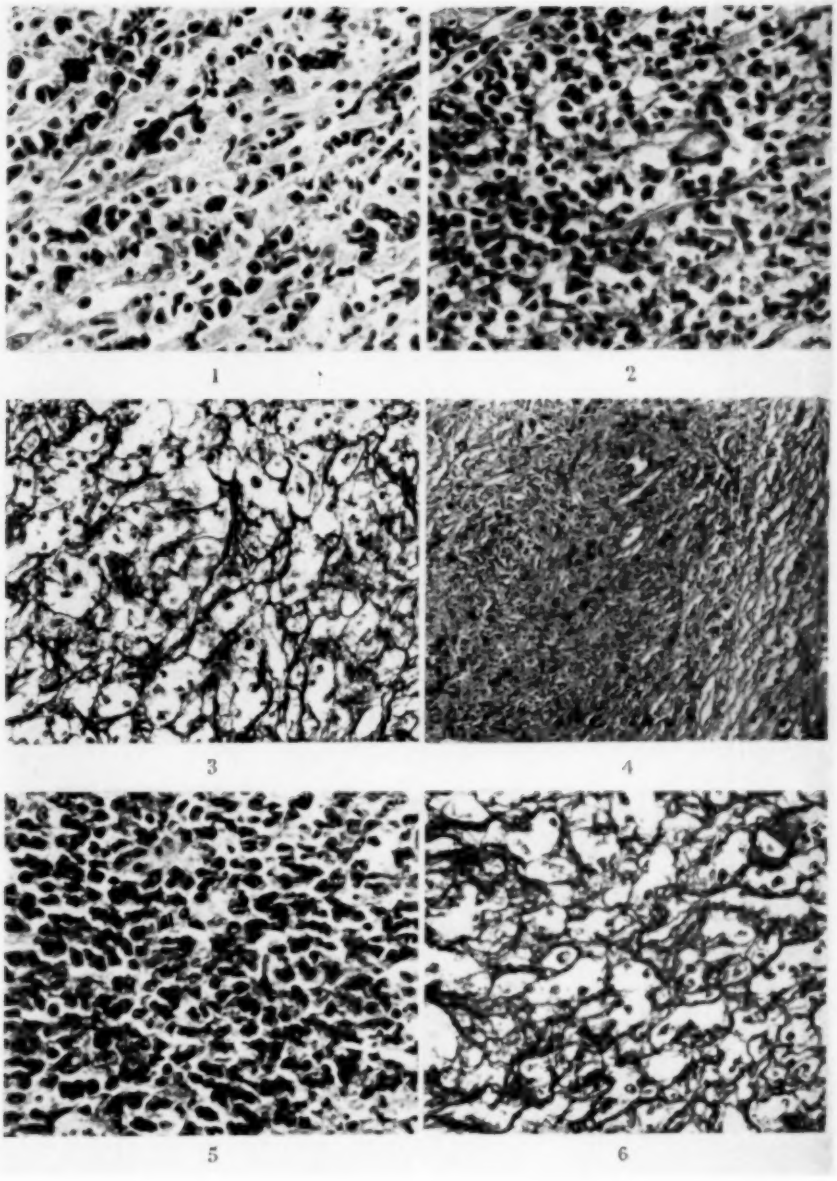
FIG. 5.—Case 2. Mesenteric lymph node showing replacement by a reticulum cell sarcoma. (H. and E. × 330).

FIG. 6.—Case 2. Mesenteric lymph node showing reticulum fibres in a reticulum cell sarcoma (Gordon and Sweets Reticulum Impregnation × 330)

FIG. 7.—Case 2. Terminal ileum, caecum and ascending colon showing thickened wall infiltrated with tumour.

FIG. 8.—Case 2. Vermiform appendix invaded by reticulum cell sarcoma. (H. and E. × 375).

FIG. 9.—Case 2. Mucosa and submucosa of terminal ileum showing invasion by a reticulum cell sarcoma. (H. and E. × 95)



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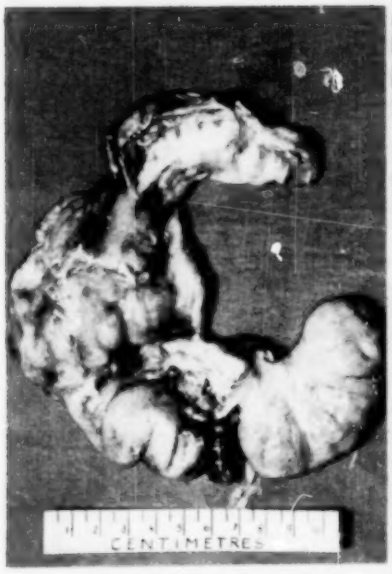

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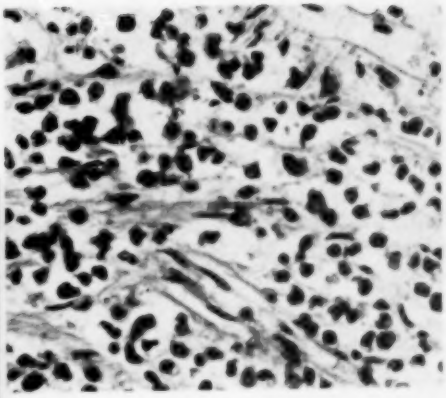

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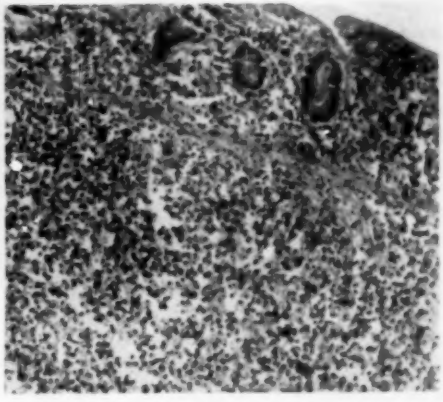

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also invaded the bowel wall in the region of terminal ileum, caecum and ascending colon. The tumour had a similar appearance in all sections. There was much necrosis and the greater part of the tissue was replaced by amorphous eosinophilic material (Fig 4). The majority of cells showed degenerative changes with loss of cytoplasm but in some areas where they were relatively intact they could be seen to have lobulated hyperchromatic nuclei and a moderate amount of eosinophilic cytoplasm. Mitotic figures were numerous. All areas including the amorphous material could be shown to contain a dense network of reticulin fibres with very little collagen present (Fig. 3). The appearances were those of a reticulum cell sarcoma.

Case 2

M.B. was born on November 20, 1955. When admitted to Auckland hospital in August 1958 there was a history that this child was fretful, tired and losing weight. He seemed to complain of intermittent abdominal pain without vomiting and the bowel motions had been normal. He had rejected rough food for about four months. On examination the abdomen appeared a little swollen and there was tenderness in the right lower quadrant. There was resistance to palpation and slight dullness on percussion. On August 18, 1958, a laparotomy was performed. The surgeon, who had not operated on the elder brother was aware of the family history and he read the case notes and post mortem report on L.B before the operation. He reported very similar findings—a large, hard, irregular. mass which appeared to arise in the hepatic flexure and spread to invade the psoas muscle, right kidney, mesenteric and para-aortic nodes. Two mesenteric lymph nodes were examined histologically and one of these showed the structure of a reticulum cell sarcoma (Fig. 5 and 6). This patient was given small doses of deep X-ray therapy receiving 1,500 r. in all. He did improve a little and the mass appeared to get smaller. On November 6, 1958, an examination was made under general anaesthesia and the tumour was found to occupy the right half of the abdomen from the right costal margin to the iliac fossa.—This patient belonged to blood group AB and was rhesus negative. The white blood count was not recorded higher than 10,000 per cu. mm. with 91 per cent neutrophils. The haemoglobin fell as low as 6·9 g. He lived about three months after the diagnosis was established and continued to lose weight and died on November 13, 1958.

Post mortem findings.—The autopsy was performed 18 hours after death and a full examination was carried out. There was a thrombus attached to the wall of the lower part of the inferior vena cava and partially occluding it. Peritonitis was present and this had been caused through a perforation of the small bowel near the ileo-caecal region. The main features were in the alimentary tract. Fibrinous exudate covered a large part of the small and large intestine. There were many adhesions of the small bowel on itself and to the parietal peritoneum. Most of these could be broken down with pressure but some around the right iliac fossa were very firm. The terminal ileum, caecum and lower part of the ascending colon showed a thickened wall and the appearances were those of infiltration with tumour (Fig. 7). The mucosa was intact but stretched. The ileo-caecal valve was thickened. The vermiform appendix was caught up in some adhesions and appeared to be replaced by tumour The remainder of the small and large intestines appeared normal. The liver contained a large deposit of tumour measuring 3 cm. across. This was in the lower part of the

right lobe and the hepatic flexure was adherent here. There were enlarged lymph nodes in the mesentery, at the ileo-caecal region and around the coeliac axis. The spleen was small, soft and normal, in appearance. The kidneys showed no abnormality.

Histological examination.—The tumour in the small and large intestines, lymph nodes, vermiform appendix (Fig. 8) and liver had a uniform appearance. There were masses of polyhedral cells, fairly even in size with scanty cytoplasm and prominent hyperchromatic nuclei. Some of the nuclei were crenated and others were pyknotic. Mitotic figures occurred. Much of the tumour was degenerate and in parts there was hyaline material (Fig. 9) and broad bands of collagen. Reticulin preparations showed reticulin fibres surrounding tumour cells. The appearances were those of a reticulum cell sarcoma.

DISCUSSION

Koller (1959) points out that certain types of tumour show a high susceptibility to inheritance. Amongst these tumours are retinoblastoma, neurofibromastosis, multiple polyposis and xeroderma pigmentosum. In the first three types of tumour the disease is transmitted by a dominant gene, while in the last the gene is recessive. Brief review of studies of cancer incidence in monozygotic twins show that the presence of predisposition does not decide the fate of an individual, it is the outcome of the environmental influence acting upon the genetic constitution.

Devore and Doan (1957) in a familial study of 440 carefully verified cases of Hodgkin's disease found fifteen families in which proven multiple cases of Hodgkin's disease or other lymphomata developed in blood relatives with or without direct contact and in some instances with an interval of some years between.

McConnell (1958) found a nasopharyngeal carcinoma (confirmed by lymph node biopsy) in a female child aged 8 years and her brother developed a similar tumour at the age of 8 years. Both children died of secondaries in the liver some months after diagnosis.

Howel-Evans *et al.* (1958) described an association between keratosis palmaris et plantaris (tylosis) and carcinoma of the oesophagus in two Liverpool families. Eighteen cases of carcinoma of the oesophagus have occurred. There is unequivocal evidence that the neoplasm was associated with tylosis in seventeen patients. No case of oesophageal carcinoma has occurred in members unaffected with tylosis. Tylosis is inherited as an autosomal dominant.

Herrell, Ruff and Bayrd (1958) record the occurrence of multiple myeloma in two siblings aged 53 and 55 years when first seen.

Regarding the nature of the tumour Mestel (1959) reports thirteen cases of lymphosarcoma in the young (including one of reticulum cell sarcoma) considered to be confined to the small intestine at time of diagnosis. Twenty five further cases are found in the literature. The disease is most common in the 3–8 year old group and five and a half times more prevalent in the male than the female. The prognosis is bad, the average length of survival being just over thirteen months.

Rosenberg *et al.* (1958) commenting on lymphosarcoma (which includes giant follicular lymphosarcoma and reticulum cell sarcoma) from the records of thirty years at the Memorial Centre of New York state that amongst 1269 cases

of lymphosarcoma 69 cases were 15 years of age and below. In the older children males show predominance but in the very young the ratio approaches unity. The disease in children presents with abdominal symptoms about twice as often as in adults and the bowel is often involved. In 11 cases in this series the disease was fatal as a result of rapid uncontrolled local disease before spread occurred. This picture was somewhat more common in reticulum cell sarcoma. Of 26 children on whom autopsies had been performed about three quarters had involvement of the retroperitoneal lymph nodes and just over half had foci in the small intestine. Half the children were dead in seven months. The disease seems more aggressive in childhood. Certain sites, other than in lymph nodes, appear to be the primary focus more frequently in children than in adults.

In this particular family the parents of the siblings described were anxious to ascertain whether they could have further offspring with impunity and at present have decided against it. Dr. Fraser Roberts (1958, personal communication) was consulted and he wrote that he considered it unlikely that the condition is due to a chance coincidence. A single gene can sometimes do what is usually determined by a non-genetic error of development. There is difficulty in giving any genetic prognosis but the risk of recurrence on a further child is probably appreciable. The worst it could be would be one in four (due to a recessive gene) but the genetics might be more complicated and the more complex the genetics the better the outlook in terms of odds.

SUMMARY

The cases of two brothers who died in the third year of life from a reticulum cell sarcoma are described. The diagnosis was established during life, in each case by biopsy and in the second case by exploratory laparotomy.

Full autopsies were carried out on both cases, the tumour affecting the terminal ileum, caecum, ascending colon and lymph nodes.

The heredity nature of tumours is briefly discussed. The question arises as to what advice can be given to the parents as to the possibility of any further offspring being similarly affected.

Malignant lymphomata in children are briefly considered.

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ANAEMIA AND POST-CRICOID CARCINOMA

A. JACOBS

From the Pathology Department, St. Mary's Hospital, London, W.2

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THE association between chronic anaemia and post-cricoid carcinoma has been recognised since the observations of Kelly (1919). Atrophy of the mucous membrane of the mouth and pharynx occurs in some cases of chronic anaemia and it is generally assumed that when malignant change in the buccopharyngeal epithelium supervenes it is because this atrophy is itself a pre-malignant condition (Ahlbom, 1936; Welin, 1953; Boyd, 1961). There is little direct evidence for this assumption and the present investigation shows that it is not entirely justified.

A previous investigation of the mucous membrane of the mouth in anaemic subjects showed thinning of the epithelium in some cases, resulting primarily from atrophy of the rete pegs (Jacobs, 1960, 1961*a*). This thinning was more frequent and more severe in megaloblastic anaemia due to vitamin B_{12} deficiency than in iron deficiency anaemia, and in neither type of anaemia was it related to the patient's haemoglobin concentration, serum iron level, serum vitamin B_{12} level, or the amount of stainable iron in the bone narrow. Occasional specimens in the iron deficient group showed the formation of mature keratin. The normal squamous epithelium of the mouth contains abundant glycogen but this was either deficient or totally lacking in the atrophic epithelia found in some cases of anaemia. An increased concentration of sulphydryl groups was also found in a few anaemic epithelia, especially in the superficial zone, and this was thought to denote a tendency to parakeratosis. The same specimens also contained an abnormal polysaccharide in the superficial layers of the epithelium. There were no histological features specific to the epithelium in iron deficiency.

The present paper reviews 93 cases of post-cricoid carcinoma seen at St. Mary's and the Royal Marsden Hospitals, with particular reference to the presence of anaemia and to the histology of the epithelium adjacent to the growth. For purposes of comparison 118 cases of carcinoma occurring elsewhere in the hypopharynx are also reviewed. An attempt is made to compare epithelial histology in the mouth and post-cricoid region in anaemic patients.

MATERIAL AND METHODS

1. Ninety-three cases of post-cricoid carcimona and 118 cases of other hypohypopharyngeal carcinoma were reviewed. Some clinical details are given in Table I. In 34 of the post-cricoid cases and in 52 of the other hypopharyngeal cases sections of normal mucosa adjacent to the growth were available for study. In 18 and 22 cases respectively spare tissue was available for further sections to be cut. These were stained for glycogen and diastase resistant polysaccharides using the periodic acid-Schiff (PAS) reaction and sulphydryl groups using a

TABLE I.—*Hypopharyngeal Carcinoma: Sex Incidence and Incidence of Anaemia*

	Post-cricoid	Other sites
Number of cases	93	118
Male	10	89
Female	83	29
History of iron deficiency anaemia	29	6
History of pernicious anaemia	5	0

modified dihydroxy-dinaphthyldisulphide (DDD) method as in previous investigations (Jacobs, 1961*a*).

2. Specimens of post-cricoid mucosa were taken post mortem from 22 subjects who had not been suffering from any haemopoietic or gastro-intestinal disorder and in whom the oesophagus and pharynx appeared normal. Paraffin sections of these specimens were stained with haematoxylin and eosin, and by the PAS and DDD methods mentioned above.

3. Suction biopsy specimens were taken from both the buccal mucosa and the post-cricoid mucosa in 18 patients suffering from anaemia. A heterogenous group was selected: iron deficiency anaemia 8, megaloblastic anaemia 5, dimorphic anaemia 2, uraemia 1, chronic infection 1, carcinoma of the hypopharynx 1. Paraffin sections were stained with haematoxylin and eosin.

RESULTS

Only one of the specimens of mucosa examined showed any gross histological abnormality. This was from a case of post-cricoid carcinoma and it showed marked keratinisation.

Epithelial thickness

The epithelium of the pharynx is thinner than that found in the buccal cavity. At the latter site it is rare to find epithelium less than 300 μ in thickness in normal subjects (Jacobs, 1960). Measurement of the specimens from 22 normal post-cricoid mucosae showed values of 115 μ to 470 μ (Table II). Comparison of the maximum epithelial thickness in the oral and post-cricoid mucosae of 18 anaemic subjects indicated that the two tend to be related (Fig. 1). Some of the epithelia at both sites were abnormally thin.

The maximum epithelial thickness of every specimen of mucosa found in relation to a hypopharyngeal carcinoma was measured. The results are shown in Table II. There was no difference between the thickness of the normal epithelia

TABLE II.—*Maximum Thickness of Pharyngeal Epithelium*

Type of patient	Number of cases	Mean epithelial thickness (μ)	Standard error	Range (μ)
Normal	*22*	*208*	*5·4*	*115–470*
All carcinomas	*103*	*204*	*2·8*	*85–565*
Post-cricoid carcinomas	42	218	4·3	95–500
Other carcinomas	61	196	3·5	85–565
Carcinomas with preceding anaemia	19	216	6·3	85–375
Carcinomas with no preceding anaemia	84	203	2·9	95–565

and those from patients with carcinoma. Neither the epithelia from cases of post-cricoid carcinomas nor those from other hypopharyngeal carcinomas when grouped separately showed any significant deviation from the normal. Cases were also grouped into those with a history of anaemia and those with no such history ; neither of these groups showed any significant difference from the normal.

Glycogen

Glycogen was demonstrated as a PAS positive material which is removed by digestion with salivary amylase. Abundant glycogen is found in the normal buccal epithelium (Jacobs, 1961*a*) and is also found in the normal pharyngeal epithelium. In the present series of normal pharyngeal specimens the glycogen

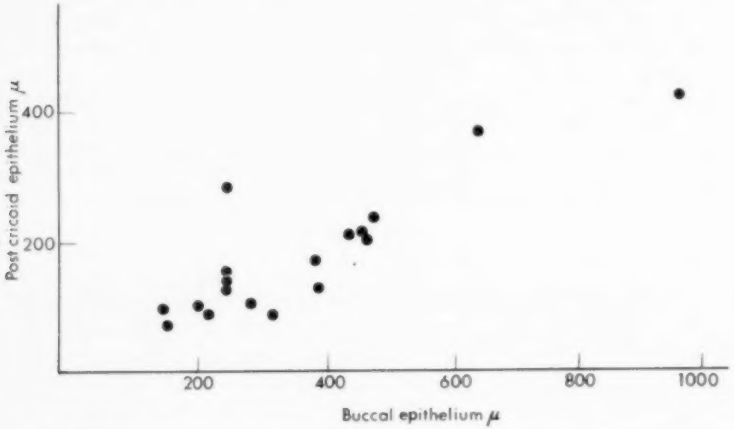

FIG. 1.—Comparison of buccal and post-cricoid epithelial thickness in 18 anaemic subjects.

content appeared to be reduced in some cases, occasionally to nil. This was due to post mortem glycogenolysis and for this reason no attempt was made at a quantitative assessment of the amount present nor was a direct comparison made with the abnormal mucosae all of which were fixed immediately after removal from the body.

Of 33 specimens of epithelium adjacent to hypopharyngeal carcinoma examined for glycogen it was absent, or present only in trace amounts, in 7. There was no significant difference between post-cricoid and other hypopharyngeal carcinomas or between those cases with a history of anaemia and those without (Table III).

TABLE III.—*The Presence of Glycogen in Pharyngeal Epithelium*

Type of patient	Glycogen present	Glycogen absent
All carcinomas	33 (83%)	7 (17%)
Post-cricoid carcinomas	16 (89%)	2 (11%)
Other carinomas	17 (77%)	5 (23%)
Carcinomas with preceding anaemia	9 (90%)	1 (10%)
Carcinomas with no preceding anaemia	24 (80%)	6 (20%)

Non-glycogen polysaccharides

In the normal buccal epithelium a substance giving a positive PAS reaction and resisting diastase digestion is found in the intercellular spaces, especially in the superficial layers (Jacobs, 1961*a*). A similar substance is found in the oesophagus (Wislocki, Fawcett and Dempsey, 1951) and the present series of 22 normal post-cricoid epithelia presented a similar picture. In a few buccal epithelia taken from anaemic patients small amounts of an intracellular PAS positive substance, resistant to diastase, were found in the superficial squames (Jacobs, 1961*a*). A trace of this substance was found in one of the series of normal post-cricoid mucosae and it was present in 7 out of 33 specimens from cases of hypopharyngeal carcinoma. It was present equally in specimens from post-cricoid and other hypopharyngeal carcinomas and in cases with and without a history of anaemia (Table IV). It was not related to the absence of glycogen from the epithelium.

TABLE IV.—*The Presence of Diastase Resistant Polysaccharide in Pharyngeal Epithelium*

Type of patient	Polysccharide present	Polysaccharide absent
Normal	*0*	*22 (100%)*
All carcinomas	*7 (17%)*	*33 (83%)*
Post-cricoid carcinomas	3 (17%)	15 (83%)
Other carcinomas	4 (17%)	18 (83%)
Carcinomas with preceding anaemia	2 (22%)	7 (78%)
Carcinomas with no preceding anaemia	5 (16%)	26 (84%)

The basement membrane stained a little variably with the PAS method but there was no essential difference in staining between the normal pharyngeal mucosae and those obtained from patients with pharyneal carcinoma. The appearances were similar to those found in the buccal mucosa (Jacobs, 1961*a*).

Sulphydryl groups

All the specimens of normal pharyngeal epithelium showed positive staining with the DDD method indicating the presence of sulphydryl groups. In 4 specimens from patients with carcinoma the amount of staining was greatly in excess of normal. A semi-quantitative assessment of the strength of the reaction is shown in Fig. 2 where it can also be seen that the presence of the abnormal diastase resistant polysaccharide mentioned above was usually associated with a high concentration of sulphydryl groups, a finding comparable to the state in some abnormal buccal mucosae (Jacobs, 1961*a*). The specimens showing a high concentration of sulphydryl groups were associated with carcinoma from both sites in the hypopharynx and in cases both with and without a history of anaemia.

DISCUSSION

Dysphagia occurs in 15–20 per cent of patients suffering from chronic iron deficiency anaemia (Whitby and Britton, 1957; Jacobs, 1960). The syndrome of anaemia, glossitis and dysphagia, often in association with koilonychia and angular stomatitis, occurs most frequently in women and was first described by Paterson (1919) and Kelly (1919). They paid scant attention to the nature of the

anaemia but both noted the association of the syndrome with cancer at the mouth of the oesophagus. Kelly wondered whether there was something at the site peculiar to the female sex that predisposed it to both spasm and cancer. Moersch and Conner (1926) found the dysphagia to be associated with a hypochromic anaemia, usually of some severity, and of the 10 patients whose gastric function they tested 8 were found to be achlorhydric. They noted the frequent presence of an atrophic-looking mucosa in the mouth, pharynx and oesophagus. Witts (1931) describing 13 cases of glossitis, dysphagia and anaemia in women noted that the condition was usually associated with achlorhydria, that it responded to treatment with iron but had a tendency to relapse. It is now generally assumed that the Paterson-

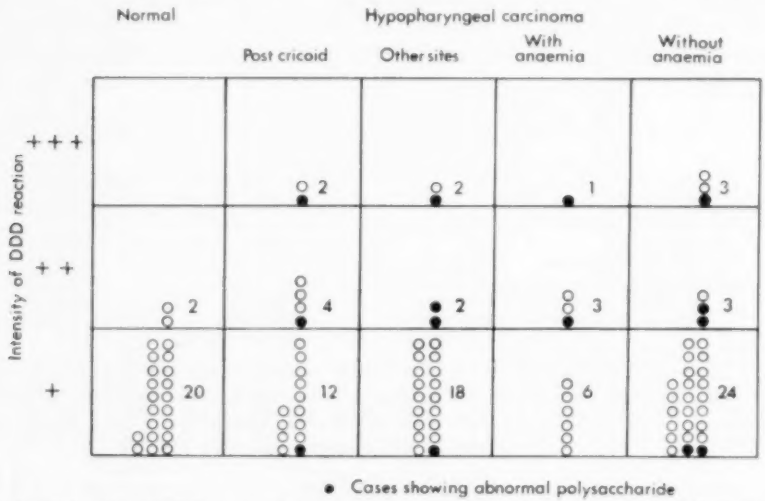

FIG. 2.—Intensity of DDD reaction for sulphydryl groups in post-cricoid epithelium from normal subjects and subjects with hypopharyngeal carcinoma.

Brown Kelly syndrome is associated with chronic iron deficiency anaemia, usually with gastric achlorhydria (Whitby and Britton, 1957). Some patients with the syndrome are found to have a web of mucosa in the post-cricoid region or in the upper oesophagus. This often remains after the dysphagia has been relieved.

When patients with anaemia and dysphagia are kept under observation squamous carcinoma of the upper alimentary tract, especially in the post-cricoid region, is found to develop with a frequency much greater than normal. Simpson (1939) found carcinoma occurring in 10 out of 18 patients in his care. These were located in the post-cricoid region (4 cases), lower oesophagus (1 case) and at the gastro-oesophageal junction (5 cases). Owen (1950) observed 28 women with anaemia and dysphagia for 15 years and during that time 5 of them developed post-cricoid carcinoma. Kirchenberger and Flett (1946) followed 100 cases of benign post-cricoid stenosis for up to 10 years but did not find any cases of carcinoma. A recent review of these cases, however, shows that 6 have developed post-cricoid carcinoma (Flett, 1961, personal communication). Ahlbom (1936)

stated that 70 per cent of his cases of carcinoma of the mouth, pharynx and oesophagus in women had either "Plummer-Vinson's" syndrome or simple achlorhydric anaemia and that in cases of post-cricoid carcinoma the incidence was 90 per cent. He also stressed the association with an atrophic mucosa. Lederman (1958) recorded the results of examining 106 patients with post-cricoid carcinoma for clinical evidence of epithelial atrophy. This was found in 60 cases and was manifested by a smooth tongue, angular stomatitis or koilonychia.

The present series of patients with carcinoma of the hypopharynx showed the characteristic sex distribution (Table I). In the post-cricoid group 89 per cent of the cases were in women while in the other hypopharyngeal group 75 per cent were in men. There was a history of anaemia in 37 per cent of the post-cricoid cases but in only 5 per cent of those with other growths. It is of some interest that 5 of the patients with post-cricoid carcinoma, all women, were said to be suffering from pernicious anaemia. Their histories were studied in some detail and in 3 cases there was no doubt as to the accuracy of the haematological diagnosis. In the other two cases details of the original blood investigations could not be found. Dysphagia in association with pernicious anaemia has been reported in the past (Jones and Owen, 1928; Croskery, 1928; McGibbon, 1935) and cases have also been seen by the present writer. Simpson (1939) reported a case of post-cricoid carcinoma in association with pernicious anaemia and Wilkinson (1950) found 11 cases of buccopharyngeal carcinoma among 1820 cases of pernicious anaemia.

Post-cricoid carcinoma is the growth most commonly associated with pre-existent anaemia and it is said that the atrophy of the mucous membrane resulting from anaemia is in fact a pre-malignant condition (Welin, 1953; Boyd, 1961). It is usually assumed to be related only to iron deficiency anaemia. The histology of the mucosal lesion in iron deficiency anaemia has received little attention in the past. Paterson (1919) in his original paper on dysphagia stated that there was thinning of the epithelium of the tongue and cheek and an infiltration of the underlying connective tissue. Suzman (1933) reported a case with thinning and keratinisation of the epithelium of the tongue and oesophagus and Savilahti (1946) described a case where the epithelium was thin over the tongue, pharynx and oesophagus. McGee and Goodwin (1938), however, referred to a case in which the oesophageal mucosa was neither thin nor keratinised. Palmer (1952) stated that there was thinning of the epithelium, leukoplakia, focal destruction of the muscularis mucosa and atrophy of the muscularis propria in the oesophagus.

A systematic examination of the histological changes in the buccal epithelium of anaemic patients was recently carried out (Jacobs, 1960, 1961*a*); it was found that thinning of the epithelium occurred in some cases together with a reduction or disappearance of glycogen from the prickle cells. Abnormal keratinisation and some evidence of parakeratosis were also found. All these changes appeared to be more frequent and more marked in cases of megaloblastic anaemia, most of them being classical pernicious anaemia. It was not known whether similar changes were also occurring in the pharyngeal mucosae of these patients but certainly the signs of buccal epithelial atrophy were not related to the symptom of dysphagia which was complained of by 12 out of 64 patients. Cheli, Dodero, Celle and Vassalotti (1959) compared the histology of the oesophageal mucosa in hypochromic anaemia with the clinical appearance of the tongue and concluded

that atrophy at the two sites was unrelated. The present comparison of epithelial thickness in the mouth and hypopharynx in 18 cases of anaemia seems to indicate that there is some parallel between the mucosae from the two sites.

The significance of the post-cricoid or upper oesophageal web in the aetiology of post-cricoid carcinoma is uncertain. Only a minority of patients with anaemia and dysphagia have a demonstrable web but it is probable that most of these webs are associated with iron deficiency. Of 46 patients with upper oesophageal webs observed by Shamma'a and Benedict (1958) only 12 were known not to be anaemic. The occurrence of webs in the presence of occult sideropenia was recognised by Waldenstrom (1938) and similar webs in non-anaemic patients with low serum iron levels have been seen by the present author. The histology of post-cricoid webs is unremarkable. There is usually normal epithelium lying on a connective tissue stroma. Minimal evidence of chronic inflammation is sometimes present in the stroma. Carcinomas rarely arise from the web itself. In 6 of the cases reviewed by Shamma'a and Benedict (1958) they occurred in the mouth and in 3 cases in the oesophagus. Welin (1953) found 10 cases of carcinoma after keeping patients with a benign stricture under observation and in all of them malignant change had occurred either above or below the site of obstruction. Thus although the post-cricoid web is related to chronic iron deficiency and carcinoma it does not itself appear to be a pre-malignant lesion.

None of the present histological investigations reveals any difference between the appearance of the epithelium associated with post-cricoid carcinoma and that associated with carcinoma higher in the pharynx. The epithelia adjacent to all the carcinomas were within the normal range of thickness but a few did display abnormal histochemical features similar to those found in anaemic buccal epithelia. The increased concentration of sulphydryl groups and the presence of an abnormal polysaccharide found in some cases is a characteristic of some anaemic mucosae and the deficiency of glycogen is also sometimes found in the anaemic mouth (Jacobs, 1961*a*). These findings were, however, present both in cases with a history of anaemia and those without. It cannot of course be certain that the non-anaemic patients had never suffered from deficiency of a haematinic factor. Mucosal lesions due to iron or vitamin B_{12} deficiency in the absence of overt anaemia have been described (Waldenstrom, 1938; Adams, 1957). Thinning of the epithelium which is so prominent in some cases of anaemia is not found in the vicinity of pharyngeal carcinomas but this may be because of reversion to normal following treatment with haematinics. Cahn, Eisenbud and Blake (1961) examined 100 white lesions of the mouth and concluded that dissolution of the basement membrane was a distinguishing feature of pre-malignant lesions. They stated that the PAS reaction might make it possible to distinguish potentially malignant epithelium from benign epithelium. No abnormality of the basement membrane was found in any of the present series of epithelia from the neighbourhood of carcinomas, although it was considerably disrupted in the malignant areas themselves.

If the abnormalities noted near some pharyngeal cancers and associated with anaemia are indeed a facet of a pre-malignant condition resulting from anaemia it is surprising that they are equally frequent in the two types of growth. The experience of most workers is that post-cricoid carcinoma is preceded by anaemia more frequently than other squamous growths of the upper alimentary tract and this is borne out by the present series (Table I). In the present state of

knowledge a number of conditions appear to be linked together but we are in no position to determine which are the key aetiological factors. Epithelial changes occur in some cases of anaemia and similar changes may occur in relation to hypopharyngeal carcinoma ; the cause is not known in either case. It may be significant that those cases of iron deficiency preceding post-cricoid carcinoma are usually associated with achlorhydria ; thus gastric atrophy is common to all the anaemias associated with this type of cancer whether due to deficiency of iron or vitamin B_{12}. The abnormally high incidence of pernicious anaemia in the present series of post-cricoid carcinomas suggests that iron deficiency alone is not a predisposing factor. Kirchenberger and Flett (1946) showed that the return of dysphagia in anaemic patients was not influenced by treating the patients with iron.

It is not known whether there is a genetic predisposition to the development of squamous carcinoma in the pharynx. The familial incidence of gastric atrophy with associated pernicious anaemia and hypochromic anaemia is well recognised and this is the common back-ground to post-cricoid carcinoma. Simpson (1939) records the interesting history of a woman with pernicious anaemia who later developed a post-cricoid carcinoma. Her sister had died from the same type of growth some years previously and a third sister suffered from anaemia with dysphagia but refused investigation. Figures supplied by the Registrar General for England and Wales show some similarity between the distribution of post-cricoid carcinoma in women and the distribution of pernicious anaemia in various parts of the country (Jacobs, 1961*b*). There is a surprisingly high incidence of post-cricoid carcinoma in Wales but this does not appear to be due to the local prevalence of iron deficiency as Kilpatrick (1961, personal communication) has shown the serum iron levels in the South Wales population to be substantially the same as in the West Riding of Yorkshire where the mortality from post-cricoid carcinoma is less than half that in Wales.

The present investigation of epithelia has merely shown that there are some changes common to iron deficiency anaemia, pernicious anaemia and hypopharyngeal carcinoma, especially in the post-cricoid region. The nature of the fundamental lesion and its cause remain as yet undiscovered.

SUMMARY

Post-cricoid carcinoma is not usually associated with the mucosal changes found in anaemic patients even when there is a history of anaemia. In a few instances the epithelium shows histological changes similar to those in some anaemic mucosae but these changes are not related to preceding anaemia and are also found near carcinomas higher in the pharynx where a history of anaemia is uncommon.

If there is a pre-malignant lesion in anaemic bucco-pharyngeal epithelium it has not been revealed by present methods.

I am indebted to the medical staff at St. Mary's and the Royal Marsden Hospitals for granting me access to their notes and to Dr. N. F. C. Gowing for his help in allowing me to use histological material from his files. I also wish to thank Miss P. Evans for technical assistance and the Medical Research Council for a grant supporting this work.

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AN EXPERIMENTAL STUDY OF THE PATHOLOGICAL EFFECTS OF CIGARETTE CONDENSATE IN THE LUNGS WITH SPECIAL REFERENCE TO CARCINOGENESIS

J. W. S. BLACKLOCK

From the Department of Pathology, St. Bartholomew's Hospital, London, E.C.1

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It has been demonstrated (Blacklock, 1957) that malignant tumours can be produced in the lungs of rats with various carcinogens, such as benzopyrene or methylcholanthrene, and in a small number of rats with the condensate from cigarette smoke. In view of the results with the condensate, it was desirable to repeat the experiments in a larger series of animals and in addition to study the histo-pathological changes in the lungs preceding the development of malignant tumours. To ensure that there was no other carcinogen at work in the course of the experiments here described, no experimental work with known carcinogens was carried out at the time in the laboratory concerned. Further, it is most unlikely that the benzopyrene content of the air at St. Bartholomew's Hospital, during the years this study was in progress, had any carcinogenic effect on the lungs of the experimental animals used, as my colleague, Dr. P. J Lawther, informs me that the mean benzopyrene content during that time was 2·5 μg./100m^3 and the mean smoke concentration was 0·15 mg./m^3.

Others have reported with varying degrees of success the experimental production of tumours by painting cigarette condensate on the skin of mice (Wynder, Graham and Croninger, 1953, 1955; Wynder, Lupberger and Grener, 1956; Sugiura, 1956; Engelbreth-Holm and Ahlmann, 1957). Similar experiments on mice extending over 18 to 19 months by Passey *et al.* (1954), Gwynn and Salaman (1954), Hamer and Woodhouse (1956) and Gellhorn, Klausner and Hibbert (1956) have yielded negative results. In rabbits Roffo (1937, 1939), Schürch and Winterstein (1935, 1937), Flory (1941) and Graham, Croninger and Wynder (1957) have produced tumours in the skin of the ear by the repeated application of tobacco tar, though of all these authors only Graham *et al.* used cigarette condensate, the others using distillates of various types of tobacco or of cigars. The amount of condensate used in such painting is gross and bears little relation to the amount that would be inhaled into the lung during smoking. The repeated painting over months of cigarette condensate on the skin may cause recurrent damage and even ulceration, as in Wynder's experiments, with subsequent repeated attempts at repair. This condition, at least in the human subject, predisposes to malignant change. In the experimental animal, as pointed out by Mackenzie and Rous (1941) and by Pullinger (1943), the additional factor of wound healing has a considerable effect on the yield of tumours by carcinogens. The above experiments on the carcinogenic effect of cigarette condensate on the skin of certain animals have been accepted by some and rejected by others. It remained to be proved, however, if the condensate had the same effect on the lungs. Cigarette smoke is an aerosol, the oily particles of which when first formed

vary in size from 0·3 to 1 μ. and particles of this size can, on inhalation, pass deeply into the lung. Accordingly rats and mice have been kept in an atmosphere of cigarette smoke for periods, though unlike in the human subject where the smoke is inhaled directly into the lungs through the mouth, the smoke in the animal passes through the nose and much of the smoke particles must be filtered out in the complicated nasal passages and never reaches the lungs. The results of keeping animals in an atmosphere of cigarette smoke have been varied, for example, Essenberg (1952) and Essenberg, Horowitz and Gaffrey (1955) found a greater number of papillary adenocarcinomata in pure strains of mice exposed to cigarette smoke than in the control group, and Mühlbock (1955), working with hybrid mice, found a higher incidence of lung tumours in those exposed to cigarette smoke than in the controls kept under the same conditions but not exposed to the smoke. Passey (1955 and personal communication) was unable to produce tumours in the lungs of hamsters or of rats exposed to cigarette smoke for periods of 12 to 20 months, and Lorenz *et al.* (1943) found the average number of neoplasms the same in the control mice as in those of the same strain exposed to tobacco smoke for 250 days. Campbell (1936) on the other hand found a slightly higher incidence of tumours in the lungs of mice exposed to tobacco smoke than in the controls, the tumours being more frequent about the end of the second year. Thus the results of experimental work in animals with cigarette condensate have so far been conflicting.

The preparation of the cigarette condensate

The condensate was prepared by smoking cigarettes of a popular brand and reputed to be composed of 60 per cent Virginian and 40 per cent Rhodesian tobacco in a smoking machine described by Cooper and Lindsey (1955). The condensate from 500 cigarettes was collected in acetone, which was evaporated at 50° C. and the viscous condensate was stored in a tightly stoppered glass bottle in the dark at 4° C. The chemical composition of condensate prepared in this manner and from the same brand of cigarettes has been reported by Cooper and Lindsey (1955) and contained 4 μg. of 3,4-benzopyrene per 500 cigarettes. In the experiments described here the nicotine was not removed as the chemical treatment involved might cause changes in the condensate.

Operative procedure

For most of the experiments male white rats 8 to 12 weeks old were used. These rats were all of the same strain and were bred at the Chester Beatty Institute in London. The same type of rat was used in the work reported in 1957. Guinea-pigs and rabbits were used for some experiments. The former were 3 to 4 months old and the latter 5 to 6 months when inoculated. Neither were of pure strains and were purchased from dealers.

In order to imitate human smoking the pure cigarette condensate prepared as above but warmed to reduce its viscosity was, at the outset of these experiments, injected down the trachea by means of a long needle with a blunt tip which reached to about the level of the bifurcation of the trachea of the rats. Most of the animals, however, died immediately of suffocation and intoxication or later from a septic pneumonia. As the mortality was so great this experiment was abandoned.

It was then decided, as in the 1957 experiments, to inject the condensate directly into the lungs after a thoracotomy had been performed. The needle having pierced the lung was directed towards the hilum in the hope that some of the condensate might gain access to a bronchus. When the condensate made as described above was warmed to allow it to pass easily through a fine needle and inoculated into the lung, much of it ran back through the needle track into the pleural sac and there were also symptoms of intoxication. It was obvious that some vehicle was required which would mix with the condensate, not alter it chemically and be of a consistency that would go through a fine needle and retain the condensate at the site of inoculation. It was found by experiment that eucerin* fulfilled these conditions and further this substance produced little reaction in the tissues. The eucerin also partly neutralised the toxic effect of the nicotine, probably because the alkaloid was liberated slowly from the mixture. When required for inoculation the appropriate amount of condensate was mixed with eucerin so that each 0·2 ml. of the mixture contained the condensate from approximately 40 cigarettes. Unless otherwise stated this amount was inoculated into the left lung of the rats, guinea-pigs and rabbits used for these experiments. In the rats submitted to this operation there was a 10 per cent mortality within 24 hours.

The condensate was used for inoculation within a few days of preparation and any remaining 15 days after preparation was discarded. But having used eucerin as a vehicle, it was desirable to test, as is reported later, what reactions it produced when introduced into the tissues and also to find if it had any carcinogenic effect.

It was realised that 0·2 ml. of the condensate-eucerin mixture was a large amount to inoculate into the lung, but it appeared from the results of the 1957 experiments that cigarette condensate was a weak carcinogen and, therefore, it was desirable to use a large dose and to allow it to act for as long as possible, that is, until the animal died or required to be killed on account of illness. Unlike in the painting of the skin with cigarette condensate, there was no repeated trauma which might be a factor in the production of a malignant tumour. In these lung experiments damage to the tissues occurred only once and was, as is afterwards shown, quickly followed by repair. If a tumour developed at the site of inoculation in the lung this could be considered as good evidence that the cigarette condensate was the cause.

Plan of experiments

It was desirable to study firstly the histopathological reaction of the the lung to cigarette condensate in animals killed at various intervals and secondly any carcinogenic effect of the condensate in animals allowed to live their entire life span. These two studies, though closely related, are now considered separately.

I. *The Histopathological Changes in the Lungs Caused by Cigarette Condensate*

These changes were studied in (a) rats, (b) guinea-pigs and (c) rabbits into the left lung of which 0·2 ml. cigarette condensate-eucerin mixture representing about 40 cigarettes was inoculated. As controls 0·2 ml. eucerin alone was in-

* The composition of the (hydrous) eucerin used in these experiments is described under the heading of Oily Cream or Unguentum Aquosum in *British Pharmacopoeia* 1958, p. 715.

oculated into the lungs of a series of rats. As a large amount of cigarette condensate was required for these experiments, Dr. H. R. Bentley of the Imperial Tobacco Company kindly supplied me with some condensate from the same brand of cigarettes as had been used in the preparation of the condensate in this laboratory. Dr. Bentley's condensate had been prepared in the automatic smoking machine described by Iles and Sharman (1957). About half the animals in this part of the work were inoculated with this condensate, the other half with that prepared in our own smoking machine as described above.

(a) *Rats*

The rats were killed at intervals of one week to over two years after inoculation. The number of animals killed at each interval varied slightly. The results produced depended on whether the condensate did or did not penetrate into the bronchial system. Owing to the experimental difficulties attending the inoculation when the thorax was open the condensate did not always enter the bronchi but sometimes remained in the lung around the air passages. When this occurred the inoculum was quickly encapsulated by granulation and later by fibrous tissue. With the phagocytosis and the solution of the eucerin used as a suspending agent, some small cyst-like spaces with fine fibrous walls resulted and in these brownish-red portions of condensate were seen. In such areas there was a slight accumulation of mononuclear leucocytes, some foam cells and a few giant cells (Fig. 1). Later some calcification took place and these areas were always sharply demarcated by a fibrous capsule from the surrounding tissues. Where, however, the condensate did gain access to the bronchi, the sequence of changes now to be described were noted.

1st week.—There were 13 rats in this group. Around the inoculum in the first few days there was a moderately acute reaction composed of polymorphs and some foam cells; later there were more mononuclear leucocytes. Some of the capillaries which the inoculum had surrounded showed thrombosis. Where the inoculum had penetrated into a bronchus there was a reaction composed of polymorphonuclear and mononuclear leucocytes. The lining epithelium showed necrosis or had lost its columnar ciliated character and was becoming more cubical in shape or even flat and was several cells thick.

2nd week.—There were 14 animals in this group. It was during this week that squamous metaplasia and less often papillomatous change was noted in the bronchi into which the inoculum had penetrated. These changes were noted in 6 out of 14 animals killed at this time. The squamous change which involved the epithelium lining the whole circumference of the bronchus was sometimes fairly regular (Fig. 2) though several layers thick; at other times it was more hyperplastic and epithelial processes extended into the underlying tissue, suggesting an early invasive tendency (Fig. 3) or where the hyperplasia was very marked giving rise to a condition not unlike what has been termed "carcinoma *in situ*" in man (Fig. 4). Papillomata were observed in the bronchi to which the condensate had gained access and were usually covered by a layer of cubical epithelium. The appearance of the metaplastic squamous change at such an early period after inoculation was remarkable. It was not until the seventeenth day after exposure to smoke of 6 to 8 cigarettes daily that Leuchtenberger, Leuchtenberger and Doolin (1958) observed atypical basal-celled hyperplasia in the bronchial epithelium of CF_1 albino mice. At a later date they observed

epithelial dysplasia similar to " carcinoma *in situ* " in man as well as squamous metaplasia in a small number of mice. Rockey *et al.* (1958) by applying cigarette condensate to the medial surface of the left main bronchus, through an artificial tracheo-cutaneous fistula, noted transitional metaplasia in 2 dogs which had received 2 treatments each and which had survived for 2 and 3 days respectively, and squamous metaplasia in other 2 dogs which survived for 17 and 25 days and received 6 and 17 treatments respectively.

3rd week.—There were 7 animals in this group and the changes were similar to those in the second week though the proportion of cases showing squamous metaplasia was less and those found were more regular and less hyperplastic than formerly. The leucocytes around the inoculum were much less numerous than formerly and composed mostly of large mononuclear leucocytes.

4th–16th weeks.—There were 36 rats in this group and during this period the rats killed at weekly intervals showed a progressive increase of fibrous tissue around the inoculum. Phagocytosis of small particles of condensate by large mononuclear leucocytes was noted now with increasing frequency. These tar-laden phagocytes were passing out from the area of the inoculum into the surrounding lung tissue. Squamous metaplasia of the bronchial epithelium, or of the lining of large cysts, derived from bronchi which had been blocked with condensate or papillomata and later with desquamated epithelium, were much less frequent and papillomatous growths into the bronchi were not so common (Fig. 5). In a cross section of a small bronchus these papilloma almost completely occluded the lumen and the central stroma of the papilloma sometimes contained small portions of condensate around which there was a mononuclear cell reaction and some small giant cells (Fig. 6). Large cystic spaces, due to the blockage of bronchi, were occasionally noted and into these papillomata, partly covered with squamous epithelium, had grown (Fig. 7). In some cases there was evidence that the condensate, where small in amount, was being absorbed and where this had been in contact with the lining of a bronchus, the epithelium of which was undergoing metaplastic change, there was now some evidence that the cells were becoming more cubical in shape. In such a bronchus one could find all three types of epithelium, normal columnar ciliated, cubical and squamous. This reversal of the metaplastic change was noted from the 12th week after inoculation onwards. This was in marked contrast with what had been observed in the earlier weeks where bronchi containing condensate were lined by atypical epithelium of one type, basal, transitional or squamous.

17th–52nd weeks.—There were 72 rats in this group, 2 being killed at weekly intervals. During this period the fibrosis and mononuclear and giant cell reactions around the condensate remained unchanged, though in some cases there was a deposit of calcium salts in the inoculum and phagocytosis of small particles of the condensate was still frequently observed (Fig. 8). Some of the larger cysts of bronchial origin containing condensate had a squamous lining in which there were localised areas of hyperplasia which was invading the tissues outside (Fig. 9). The proportion of bronchi showing squamous metaplasia was less than in the earlier weeks.

53rd–104th weeks.—There were 34 animals in this group, 2 being killed every 3 weeks. Two years after inoculation, the site of injection of 0·2 ml. of tobacco condensate was still easily recognisable naked-eye, the lesion being very localised in the lung and sometimes showing an early deposit of calcium in the condensate

EXPLANATION OF PLATES

FIG. 1.—Rat killed 14 days after inoculation. Encapsulation of brownish-red cigarette condensate by granulation tissue, mononuclear leucocytes and foreign body giant cells. ×95.

FIG. 2.—Rat killed 12 days after inoculation. Squamous metaplasia in epithelium lining the whole circumference of small bronchus. ×30 and ×110.

FIG. 3.—Rat killed 13 days after inoculation. Remains of fragments of condensate in spaces above a small bronchus showing irregular hyperplastic squamous metaplasia extending into surrounding tissue. ×55.

FIG. 4.—Rat killed 14 days after inoculation. Fragments of condensate and leucocytes in lumen of bronchus showing marked hyperplastic squamous metaplasia resembling "carcinoma *in situ*". ×55.

FIG. 5.—Rat killed 56 days after inoculation. Papilloma growing into small bronchus. In the stroma of the papilloma are spaces which contained condensate. Multiplication of the basal cells is present both in the epithelium covering the papilloma and to a lesser extent in the bronchus. ×65.

FIG. 6.—Rat killed 56 days after inoculation. Cross section of a small bronchus, partly occluded by a papilloma, cut transversely. In the stroma of papilloma are spaces from which the condensate has been absorbed or dissolved and around these are some small giant cells. ×105.

FIG. 7.—Rat killed 112 days after inoculation. Part of cross section of a distended bronchus in which fragments of condensate are still present. Extending into the lumen is a papilloma partly covered by columnar ciliated epithelium and partly by squamous. The wall of the bronchus to the right of the papilloma is lined by squamous epithelium. ×50.

FIG. 8.—Rat killed 166 days after inoculation. Mononuclear leucocytes near site of inoculation which have phagocytosed small darkly staining particles of condensate. ×275.

FIG. 9.—Rat killed 263 days after inoculation. Part of a bronchus distended with condensate and squame cells. Squamous metaplasia of lining epithelium with extension into the peribronchial tissue. ×80.

FIG. 10.—Rat killed 491 days after inoculation. Part of a bronchus distended with condensate and squame cells. Squamous metaplasia of lining epithelium, extension into peribronchial tissue and formation of epithelial perles. ×95.

FIG. 11.—Rat killed 570 days after inoculation. Part of wall of distended bronchus showing remains of dark condensate (at top). Squamous epithelioma infiltrating lung tissue. ×50.

FIG. 12.—Guinea-pig killed 7 days after inoculation. Small bronchus with some condensate and leucocytes in lumen. The lining epithelium shows early squamous change. ×165.

FIG. 13.—Guinea-pig killed 13 days after inoculation. Portion of a bronchus distended with condensate (at the top) and some leucocytes. The lining epithelium has all undergone squamous metaplasia. ×72.

FIG. 14.—Guinea-pig killed 21 days after inoculation. Portion of the lumen of a distended bronchus containing condensate and leucocytes. Numerous papillomata covered with a cubical epithelium are growing from the wall. ×60.

FIG. 15.—Guinea-pig killed 15 days after inoculation. Adenomatous formation at the edge of site of inoculum. The lining cells show much irregularity and mitotic figures are present in some. ×169.

FIG. 16.—Guinea-pig killed 275 days after inoculation. Papillomata growing into a bronchus distended with condensate to show the cubical character of covering epithelium. ×130.

FIG. 17.—Guinea-pig killed 285 days after inoculation. A sessile papilloma growing into a distended bronchus. The growth is covered with a very hyperplastic squamous epithelium and in the stroma are numerous adenomata. ×35.

FIG. 18.—Guinea-pig killed 295 days after inoculation. Portion of one of the main bronchi with cartilage in wall showing very marked hyperplasia of the basal cells. ×50.

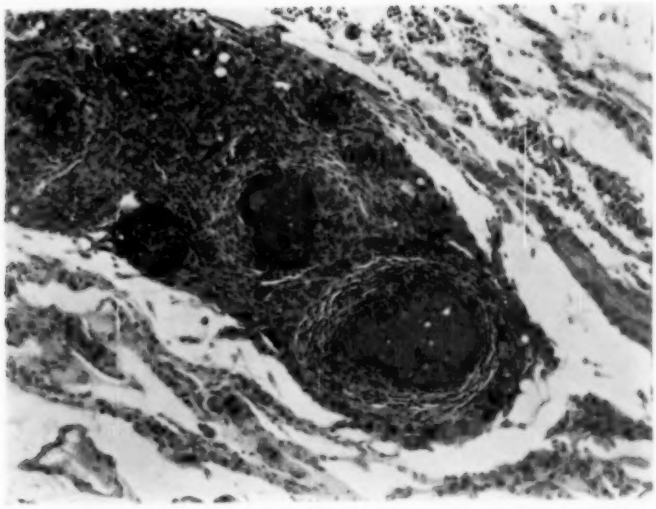
FIG. 19.—Guinea-pig killed 275 days after inoculation. Portion of wall of a bronchus showing squamous change in the lining epithelium and early sessile papilloma formation. ×50.

FIG. 20.—Rabbit killed 50 days after inoculation. Portion of wall of a bronchus with condensate in the lumen above. The lining epithelium shows marked cellular proliferation resembling "carcinoma *in situ*". ×55.

FIG. 21.—Rat died 339 days after inoculation. A pleomorphic celled cancer with an alveolar arrangement. ×210.

FIG. 22. Rat died 550 days after inoculation. A vertical section through left lung showing white tumour which is extending along peribronchial lymphatics. Dark portions of cigarette condensate are present at the centre of the growth and at the upper and lower edges. The left ventricle cut transversely is to the left and above. ×1·2.

(*continued on p.* 751)



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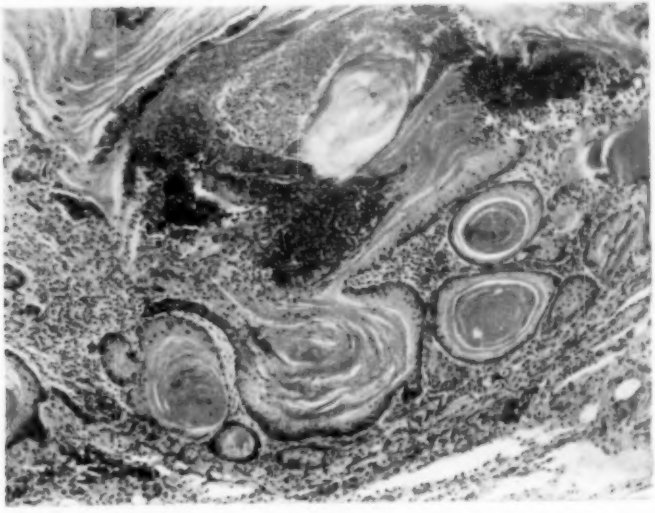

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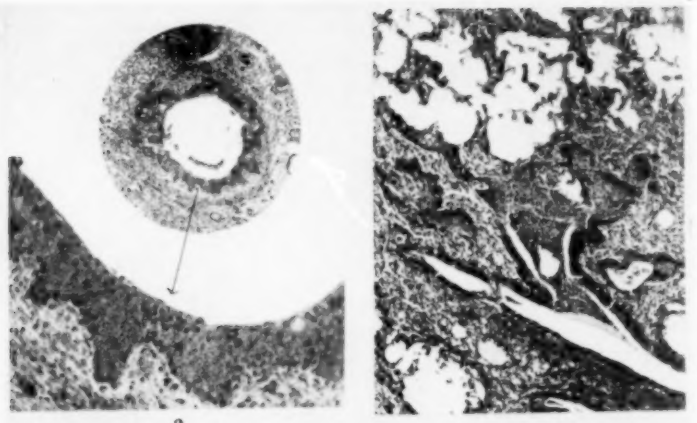

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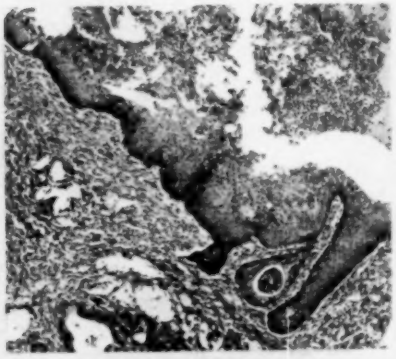

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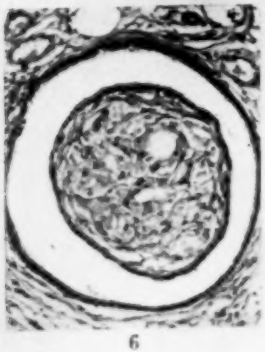

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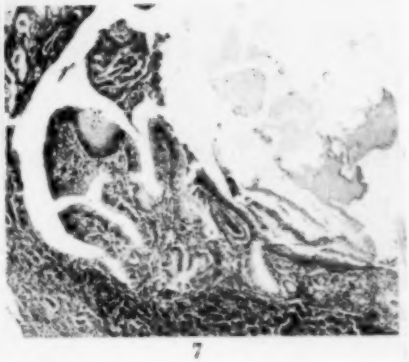

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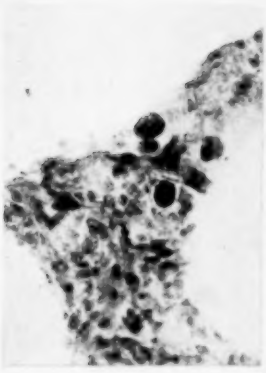

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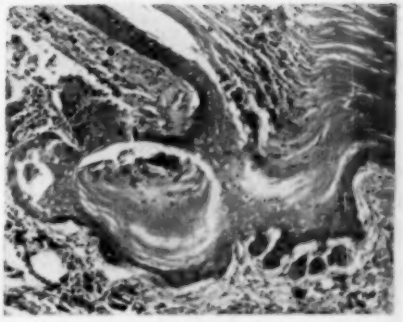

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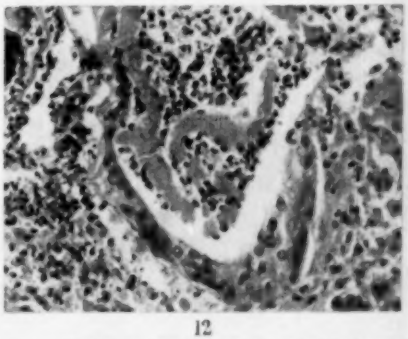

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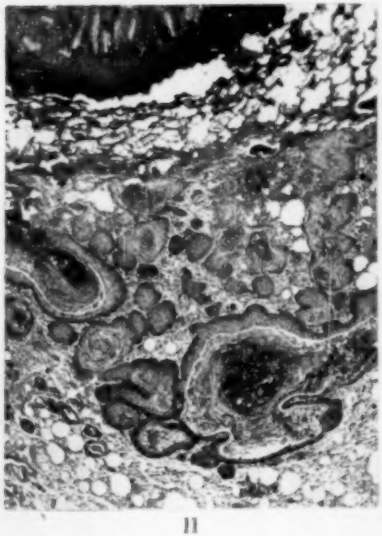

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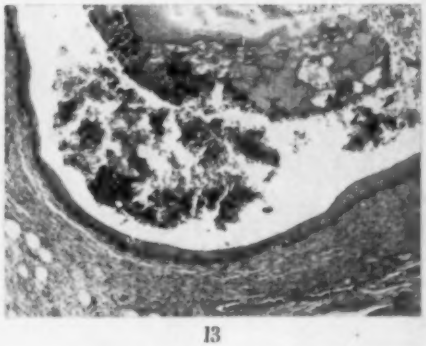

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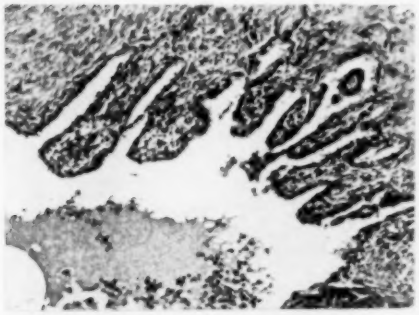

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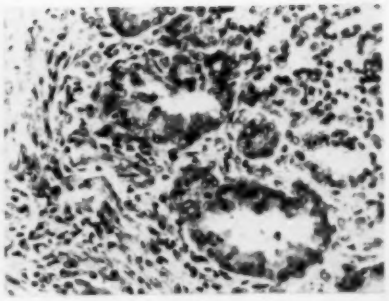

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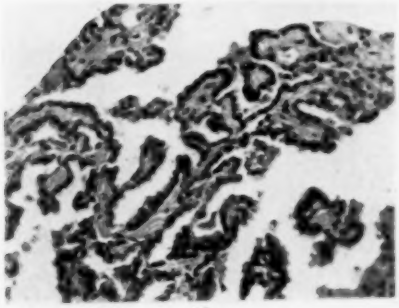

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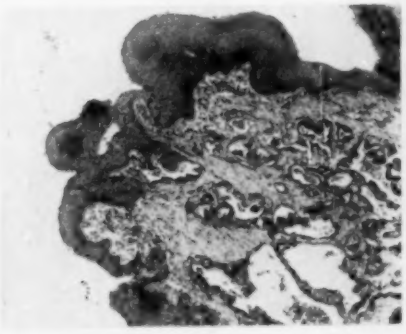

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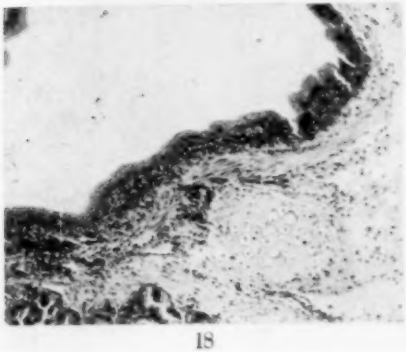

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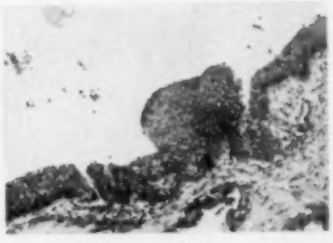

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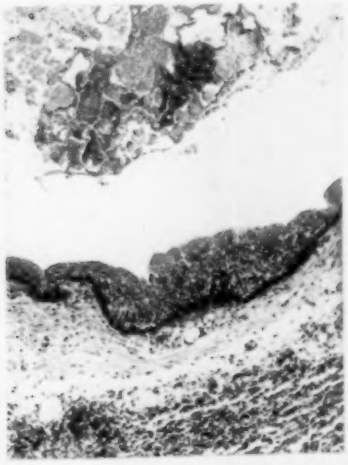

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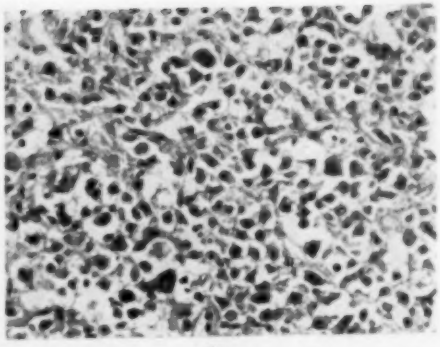

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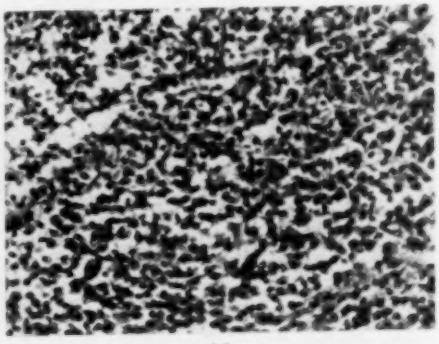

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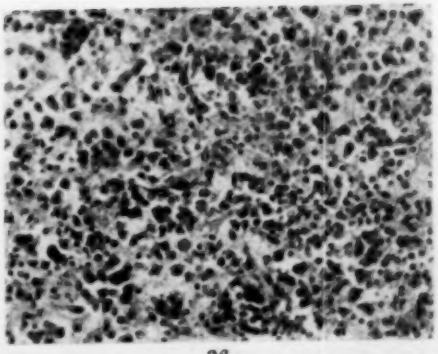

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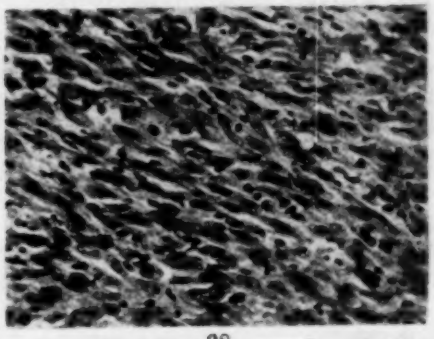

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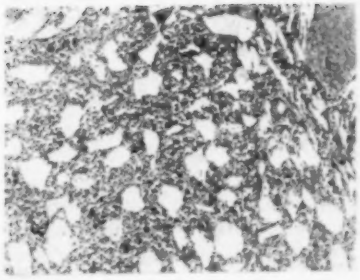

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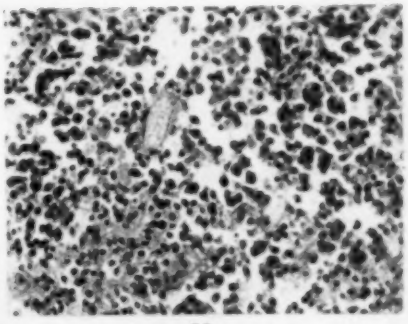

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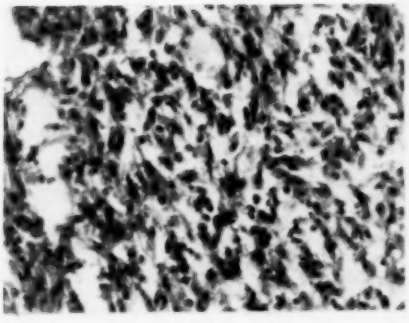

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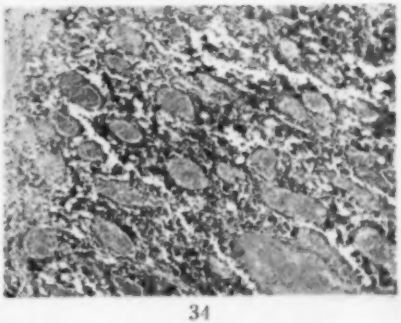

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or in the encapsulating fibrous tissue. Squamous metaplasia was still noted in the bronchi to which condensate had gained access, though less often than formerly. Owing to the blockage of bronchi by condensate or papillomata or both any metaplastic change of the normal epithelium to a squamous type had resulted in much desquamation of squame cells into the lumen which caused distension. As a result large cysts were formed containing the remains of the condensate mixed with squame cells. In some of these cysts the metaplastic epithelium was hyperplastic and extended down into the underlying tissue with the formation of epithelial perles (Fig. 10) or even invasion of the deeper tissues. In 2 animals showing such change one was killed at 491 days (70 weeks) and the other (180/58) at 570 days (81 weeks) and it was debatable on first examination if the condition was definitely malignant. With the study of further sections the first of these has been regarded as non-malignant, though it might have become so if the animal had been allowed to live. The second, however, in addition to more formation of epithelial perles showed definite invasion in all the sections examined and has been classified as an early squamous epithelioma (Fig. 11).

At this period there was more evidence that in some bronchi the metaplastic change in the epithelium was being reversed as it was possible to find in the circumference of a dilated bronchus squamous epithelium, with transition to cubical and finally normal columnar ciliated. It was only towards the end of the second year that adenomatous lesions were noted in the vicinity of the inoculum in the lungs of rats.

(b) *Guinea-pigs*

As in the experiments with rats 0·2 ml. of the cigarette condensate-eucerin mixture was inoculated into the left lung after a thoracotomy had been performed.

(*continued from p.* 750.)

FIG. 23.—Microscopic appearance of gross tumour shown in Fig. 22. The tumour is composed of darkly staining small round and oat-shaped cells. ×210.

FIG. 24.—Microscopic appearance of tumour shown in Fig. 22 to show peribronchial spread. ×6·5.

FIG. 25.—Rat died 635 days after inoculation. Vertical section through tumour in left lower lobe. ×5.

FIG. 26.—Rat died 679 days after inoculation. A pleomorphic celled carcinoma composed of small and large round cells and some polygonal cells. Some cells show mitosis. ×210.

FIG. 27.—Rat died 602 days after inoculation. Vertical section through left lung almost completely replaced by whitish tumour, embedded in which are some dark fragments of condensate. Heart is to left. ×1·2.

FIG. 28.—Microscopic appearance of tumour in Fig. 27—a spindle-celled sarcoma. ×210.

FIG. 29.—Microscopic appearance of tumour in Fig. 27 showing fragments of dark condensate in tumour and in a cystic space to right. ×55.

FIG. 30.—Rat died 646 days after inoculation. Vertical section through left lung which has been replaced by a white tumour which is breaking down. Some dark fragments of condensate are visible in the tumour. The left ventricle cut transversely is above and to left. ×1·2.

FIG. 31.—Microscopic appearance of gross tumour shown in Fig. 30. A sarcoma which is growing in the inter-alveolar tissue compressing the air vesicles. ×52.

FIG. 32.—A higher power view of Fig. 31 to show the imperfect spindle-shaped character of the cells. ×190.

FIG. 33.—Rat died, aged 584 days. A round-celled sarcoma with some fine intercellular reticulin and capillary vessels. ×190.

FIG. 34.—Rat died, aged 681 days. An angio-sarcoma composed of many large vascular sinusoids between which are darkly staining round cells. ×52.

The animals thereafter were killed at intervals and the lesions studied. There was no attempt to produce tumours in guinea-pigs as these animals are comparatively refractory to carcinogenic agents and no spontaneous tumours were found in a review of the literature by Rogers and Blumenthal (1960).

1st week.—There were 16 animals in this group. The condensate had produced some localised necrosis at the site of inoculation and around this were polymorphonuclear leucocytes and macrophages. From the 5th to the 7th day, dedifferentiation of the lung epithelium was noted and on the 7th day after inoculation actual squamous change (Fig. 12), whereas in rats this was not noted until the second week.

2nd week.—There were 19 animals in this group. The cell reaction was now more mononuclear and granulation tissue showing well-developed fibrous tissue was present around the condensate. Where in contact with the condensate there was proliferation of the basal cells in the bronchial epithelium with some hyperplasia and in some cases complete squamous change was also present (Fig. 13).

3rd week.—There were 9 animals in this group. The condensate was now well encapsulated by fibrous tissue : the cellular reaction was still mononuclear, with in addition fairly numerous foreign-body giant cells. Where the condensate had come in contact with the epithelium of a bronchus, the changes noted in the second week were still evident in addition to papillomatous change (Fig. 14) and adenomata which were lined by rather anaplastic cells some of which showed mitosis (Fig. 15). In the metaplastic squamous epithelium mitotic figures were frequently observed.

4th–5th weeks.—There were 11 animals in this group. The changes were very similar to those in the 3rd week, except for the increasing number of giant cells around the lesions. Dedifferentiation and papillomatous outgrowths of the bronchial epithelium and adenoma and papilloma and squamous metaplasia were all as frequent as at the earlier periods.

6th–52nd weeks—There were 33 animals in this group. At this late period the most noteworthy changes were the increasing amount of hyperplasia of the bronchial epithelium where condensate was present. All the changes noted above were still observed, though papillomata were more frequent and larger, some being covered with cubical epithelium (Fig. 16) and others with squamous (Fig. 17). Hyperplastic changes were still observed in the bronchial epithelium such as proliferation of the basal cells (Fig. 18) and as in the rats metaplasia to a hyperplastic squamous type (Fig. 19). Giant cells phagocytosing the condensate were also frequently seen.

(c) *Rabbits*

Similar experiments to those in rats and in guinea-pigs were performed in a small series of rabbits, 0·2 ml. of cigarette condensate-eucerin mixture being introduced into the left lung after thoracotomy. All the changes were similar to those reported in the guinea-pigs, though some were noteworthy. For example, in one rabbit killed 50 days after inoculation, the epithelium of the bronchus to which the condensate had gained access showed all degrees of epithelial change. Part was normal where in contact with the condensate and part showed extreme hyperplasia of the basal cells and dedifferentiation, forming what has been described as "carcinoma *in situ*" (Fig. 20) with intermediate changes of simple dedifferentiation to a cuboidal epithelium and squamous metaplasia.

Controls

0·2 ml. of eucerin was inoculated into the left lung of a series of rats to observe the effects of this substance which had been used as a vehicle in which to suspend the tobacco condensate. The animals were killed at intervals after inoculation.

In the first week there was an accumulation of foam cells around the eucerin with slight growth of fine granulation tissue. In the second week the eucerin was contained in many small spaces separated by fine fibrous tissue. This lesion remained unaltered throughout the rest of the time the animals lived up to 691 days. In the second year slight calcification was observed in the contents of these spaces. Apart from these changes eucerin caused no other pathological changes in the lungs of rats and even where it had gained access to bronchi, the epithelium remained normal and only some foam cells were found in the lumen.

II. *The Carcinogenic Effect of Cigarette Condensate in the Lung*

The Chester Beatty strain of rats were used in this part of the work and after inoculation they were allowed to live until death or until they appeared acutely ill and required to be killed. Into the left lung of these rats, 8 to 12 weeks old, 0·2 ml. cigarette-eucerin mixture, prepared as previously described, was inoculated. For controls a series of rats of the same strain were inoculated into the left lung with 0·2 ml. eucerin alone. A number of rats which were not inoculated in the lung were examined for the presence of spontaneous pulmonary tumours.

In 72 rats which had been inoculated with cigarette condensate the experiments were suitable for analysis. Of these 8 (11·1 per cent) had developed malignant tumours—6 carcinomata and 2 sarcomata—at the site of inoculation. In the first section of this work in which the histo-pathological effects of cigarette condensate are considered, there was one rat (180/58) which when killed on the 570th day (81 weeks) after inoculation showed an early squamous epithelioma at the site of injection. This result has been excluded for the present as its inclusion would upset the incidence of tumours in this series where the animals were allowed to live their entire life span. It has, however, been included in Table II. Of the 44 control rats inoculated with eucerin alone, 1 (2·3 per cent) developed a malignant tumour—a sarcoma—at the site of inoculation. The number of spontaneous tumours found in the lungs of a series of 275 rats amounted to 4 (1·4 per cent)—one carcinoma and 3 sarcomata (Table I).

The highest incidence of malignant tumours was thus in the group inoculated in the lung with cigarette condensate. In the first year 2 out of 13 of these rats

TABLE I.—*Incidence of Malignant Tumours in Lung* (a) *After Inoculation of Cigarette Condensate or* (b) *of Eucerin and of Spontaneous Tumours*

	—12 months				—24 months				Totals	
	Number of rats	Days		Malignant tumours	Number of rats	Days		Malignant tumours	Rats	Malignant tumours
Experiment		Lived	Average			Lived	Average			
Cigarette condensate into left lung	13	182–364	277	2 (15·4%)	59	421–729	557	6 (10·2%)	72	8 (11·1%)
Eucerin into left lung .	7	114–179	141	0	37	416–690	553	1 (2·7%)	44	1 (2·3%)
Spontaneous lung tumours	80	141–364	245	0	195	399–726	555	4 (2·05%)	275	4 (1·4%)

(15·4 per cent) developed malignant tumours whereas no malignant tumours were found in the 7 rats in the control series which died in the first year and no spontaneous tumours occurred in the 80 rats which died during this time. In the second year, 6 of 59 (10·2 per cent) rats inoculated with cigarette condensate developed lung tumours, 1 out of 37 (2·7 per cent) inoculated with eucerin alone and 4 of 195 (2·05 per cent) developed spontaneous lung tumours. Most of the malignant growths were observed between 18 and 21 months in all groups (Table II).

TABLE II.—*Types of Malignant Tumours and Times of Occurrence*

	Cigarette condensate		Controls: Eucerin		Controls: Spontaneous	
Months	Tumour	Days after inoculation	Tumour	Days after inoculation	Tumour	Days lived
—12	Cancer Cancer	339 365	0	..	0	..
—15	Cancer	408	0	..	0	..
—18	0	..	0	..	0	..
—21	Cancer *Cancer Sarcoma Cancer	550 570 602 635	Sarcoma	609	Cancer Sarcoma Sarcoma	556 584 594
—24	Sarcoma Cancer	646 679	0	..	Sarcoma	681
Total	7 cancers 2 sarcoma	339–679 602–646	1 sarcoma	609	1 cancer 3 sarcoma	556 584–681

* A squamous epithelioma which occurred in the experiments in Part I and not included in Part II

Types of Malignant Growths

Rats inoculated with cigarette condensate.—The amount of condensate injected into each rat was approximately from 40 cigarettes. There were 8 malignant growths of which 6 were carcinomata and 2 sarcomata.

(1) *Carcinoma*

Rat 12/57.—Died 339 days after inoculation. The lungs showed slight bronchiectasis and in the left lung a yellowish nodule (5 × 7 mm.) was noted. On section this was a pleomorphic-celled carcinoma (Fig. 21). This tumour was in close relation to the site of the injected condensate and eucerin, which had been dissolved during the preparation of the section, and in the vicinity many foam cells were present in addition to the malignant cells.

Rat 22/57.—Died 550 days after inoculation. A small, whitish growth, 6 × 8 mm., was noted in the left lower lobe with the remains of the condensate in the centre of the tumour around the edges of which there was peribronchial infiltration (Fig. 22). Microscopically the remains of the condensate, consisting of dark brown pigment with some spaces from which the eucerin had been dissolved, were found in immediate relation to the tumour, an anaplastic cancer composed

of darkly-staining round and oat-shaped cells (Fig. 23), which were spreading in the peribronchial lymphatics (Fig. 24) forming cellular cuffs around each bronchus.

Rat 175/57.—Died 635 days after inoculation. A yellowish, white growth, 5 × 9 mm. was found occupying most of the left lower lobe (Fig. 25) and there was marked peribronchial infiltration throughout the rest of the lobe. Some condensate was found at the side of the tumour which was composed of round and pleomorphic cells—an anaplastic cancer similar in microscopic appearance to the tumour in Rat 12/57.

Rat 170/57.– Died 679 days after inoculation. In addition to slight bronchiectasis, a whitish tumour 5 × 4 mm. was present in the left lower lobe. This tumour was a very anaplastic cancer with small and large cells, many of which showed mitoses (Fig. 26). Small fragments of condensate were found in the tumour and a bronchus at the edge showed some squamous metaplasia and hyperplasia

Rat 21/57.—Died 365 days after inoculation. A large whitish tumour occupied the whole of the left lung and had invaded the mediastinum. Microscopically this was an anaplastic small-celled carcinoma similar in appearance to that in Rat 22/57. No secondary growths were noted elsewhere.

Rat 78/57. Died on the 408th day after inoculation, when a white tumour, occupying the whole of the left lower lobe was found. The tumour was an anaplastic cancer with a similar microscopic appearance to that in Rat 12/57.

These 6 growths were all very similar microscopically, being composed of small, darkly-staining polyhedral and oat-shaped cells, some of which showed mitotic figures. The less aberrant of these cells resembled the darkly-staining basal cells of the bronchial mucous membrane and may have originated from them. The cells were arranged in irregular alveoli and all tumours had invaded the peribronchial lymphatics, forming cuffs of tumour cells around the bronchi. All were anaplastic carcinomata and in some the growth was observed to be in close relation to the remains of the inoculated cigarette condensate. In one of the rats the bronchial epithelium in relation to the tumour showed squamous metaplasia, but this altered epithelium showed no evidence of invasion at the site. No evidence of metastases elsewhere were noted in any of these growths. Another cancer in Rat 180/58 killed 570 days after inoculation, observed in Part I of this paper, was an early squamous epithelioma with cell nest formation and was infiltrating the lung tissues. This was the only epithelioma found in the present study.

(2) *Sarcoma*

Rat 7/57.—Died on the 602nd day when a large white tumour, occupying the whole of the left lung was found in which small portions of the dark condensate could still be seen (Fig. 27). Histologically the growth was a spindle-celled sarcoma (Fig. 28) and in the tumour numerous particles of the inoculated condensate were present (Fig. 29). Some of the condensate lay in cyst-like spaces from which it had been partly absorbed or dissolved in the preparation of the section.

Rat 28/57.—Died on the 646th day when a large white, partly necrotic, soft tumour, occupying the whole of the left lung was found (Fig. 30). Some dark fragments of condensate were to be seen in and around the tumour. Histologically this was a peculiar tumour which was growing in the interstitial tissue between

the walls of the air vesicles (Fig. 31) and was composed of round and spindle cells (Fig. 32).

These two growths were classified as sarcoma because of the mesoblastic character of their cells, their vascularity and, unlike the cancers, they did not invade the peribronchial lymphatics.

Controls

Rats injected with 0·2 ml. eucerin into the left lung.—Of the 44 rats so injected only one developed a malignant growth.

Rat 39/57.—Died 609 days after inoculation. The whole of the left lower lobe was occupied by yellowish-white tumour. This was a pleomorphic-celled sarcoma which was very vascular with many fine capillaries and similar in histological appearance to the growth in Rat 168/57 which is described below.

Spontaneous Tumours

The lungs were examined in a series of 275 rats for spontaneous tumours. Some of these rats had not been used for any experiment, in others eucerin, cholesterol or various compounds of this substance had been injected into the muscles of the thigh. Others had been utilised to test the toxicity by intramuscular injection of various cigarette condensates either alone or suspended in various substances such as eucerin, olive oil, lard, etc. Into the thigh muscles of others had been injected penicillin or various steroid compounds or ligatures had been inserted in connection with other experiments. These animals had been kept under the same conditions as those inoculated with condensate or with eucerin alone. In only 4 (1·4 per cent) of these 275 rats were malignant tumours found. Of these growths, one was an anaplastic carcinoma and the other 3 were sarcomata.

Rat 162/57.—Was found dead on the 556th day when the right lower lobe was noted to be solid with a white tumour. Histologically this was a carcinoma composed of darkly-staining, round oat-shaped and polyhedral cells arranged in irregular alveoli and invading the peribronchial lymphatics. The histological appearance was similar to the tumour in Rat 22/57 described above.

Rat 168/57.—Lived for 584 days. When examined *post mortem* a pinkish-white soft tumour was found in the lower lobe of the right lung. Microscopically this was a sarcoma, composed of small and large round cells with many large capillaries (Fig. 33).

Rat 117/58.—Lived for 594 days. When examined *post mortem* a whitish tumour was found in the upper lobe of the right lung. Histologically the tumour was a sarcoma, composed of small and large round cells with many small capillaries. The tumour had a similar microscopic appearance to that found in Rat 168/57.

Rat 185/57.—Lived for 681 days. A pinkish-white, soft tumour was found in the lower lobe of the right lung. Histologically this was a sarcoma composed of large vascular spaces (Fig. 34) between which many small darkly-staining round and polyhedral cells were present.

Of these 4 growths, the first was an anaplastic carcinoma which showed the typical invasion of the peribronchial lymphatics. The other 3 were of meso-

blastic nature with a well-developed vascularity and showed no evidence of invasion of the lymphatic system.

DISCUSSION

Part I. *The Histopathological Changes*

That cigarette smoke inhaled into the lung is an irritant has never been in dispute predisposing to what the man in the street refers to as "chestiness". It was of some importance to find by experiment what was the sequence of pathological changes which occurred after the inoculation of cigarette condensate through a thoracotomy into the lungs of rats, guinea-pigs and rabbits. When the condensate did not enter the air passages it was quickly encapsulated like any other foreign body gaining access to the lung. On the other hand, when the inoculated condensate penetrated into the bronchial system the ciliated epithelium was quickly destroyed and this was followed in order of time by proliferation of the basal cells, squamous metaplasia, hyperplasia of the squamous epithelium, giving rise to an appearance not unlike "carcinoma *in situ*" in man and finally to definite squamous epithelioma as well as papillomata. In the experiments here reported it can be argued that these changes were due to the injury caused by the introduction of the condensate into the lungs, but where only eucerin, which was used to suspend the condensate, was introduced in a similar way, no such epithelial changes were found. As squamous metaplasia occurred in the first week in guinea-pigs and in the second week in rats after the intra-pulmonary inoculation of cigarette condensate, it can be deduced that cigarette condensate has a very rapid and deleterious effect on the bronchial mucous membrane, destroying the ciliated epithelium and thus favouring the retention, in the bronchial mucosa, of any carcinogenic substance whether that be derived from cigarette smoke or from other sources such as atmospheric pollution. Similar changes have been reported in human lungs by Auerbach *et al.* (1956, 1957) and by Hamilton *et al.* (1957) who have reported that metaplasia and other retrogressive epithelial changes in the bronchial epithelium were commoner in the lungs of smokers as compared with non-smokers. Chang (1957) also noted that basal cell multiplication was more marked, the average thickness of the bronchial mucous membrane was greater and metaplasia more frequent in smokers than in non-smokers and that the average age of maximum frequency of these changes was from 50 to 69 years of age. It must not be assumed, however, that tobacco smoke is the only cause of such changes in the human subject, as I have observed them in the lungs of children with pulmonary tuberculosis and in the subacute and chronic bronchitis following measles and other exanthemata, and smoking was not a factor in these cases.

Are these changes pre-cancerous or do they retrogress? In the experiments described there is evidence of both these sequels. In favour of the former are the increased cellularity and the presence of mitotic figures in the areas of metaplasia and eventually the occurrence of "carcinoma *in situ*" and squamous epithelioma observed in animals which had lived to the second year after inoculation. That carcinoma does occur after the inoculation of cigarette condensate into the lungs is demonstrated in the second series of experiments, though the tumours produced were composed of undifferentiated cells possibly derived from the basal cells. The conditions of experiment favoured such malignant trans-

formation, as the cigarette condensate which provoked the epithelial change in the first place remained in contact with the altered epithelium and continued to act upon it during the whole life of the animal, thus allowing any carcinogen in the condensate to act at the same site over a long period. Indeed, it is surprising that a larger number of malignant tumours were not produced as the conditions were ideal. This, however, may be evidence that tobacco condensate is not a strong carcinogen. The same sequence of changes is occasionally seen in the human subject in other sites than the lung, for example, stones will produce metaplasia in the gall bladder or in the renal pelves and with the passage of time eventually epithelioma may sometimes occur.

In favour of retrogression is the diminished frequency with which hyperplasia, squamous metaplasia and papillomata were found in animals which had lived for two years after the introduction of the condensate into the lungs. Further, in some of the older animals the cross section of a bronchus containing some condensate would show all grades of epithelial change, for example, metaplasia at one part, basal cell proliferation at another and normal columnar ciliated epithelium at another. This was not observed in animals dying at an earlier period where a cross section of a bronchus containing cigarette condensate showed complete loss of the ciliated epithelium which was usually entirely replaced by proliferated basal cells or squamous epithelium. It was not possible to estimate the incidence of any of these changes in any group of animals as the condensate inoculated into the lungs did not always gain access to the lumen of a bronchus.

All through these interval experiments particles of condensate were observed being phagocytosed by large mononuclear cells and carried away from the site of inoculation in the lymphatics. This is just what happens to dust particles inhaled into the lungs and as with dust the phagocytes may be arrested at certain places and there the condensate accumulates. Thus the carcinogenic effect of the condensate is spread throughout the lung and away from the original site of inoculation.

Part II. *The Carcinogenic Effect*

It has been established that the lungs of rats will produce tumours when acted upon over a period of time by known carcinogens, such as 3,4-benzopyrene or methylcholanthrene (Blacklock, 1957). The lungs of mice respond in a similar way as shown by Magnus (1939) with benzopyrene and by Campbell (1942) with methylcholanthrene. Therefore, if there is any carcinogen in cigarette condensate, it is possible that the lungs of rats, which were the only animals used in these long-term experiments, might respond in a similar way as with the known carcinogens. The amount of 3,4-benzopyrene in cigarette condensate, however, is too small to exert a direct carcinogenic effect having been found by Cooper and Lindsey (1955) to be 4·0 μg. per 500 cigarettes which would represent about one to 2 parts per million of actual cigarette smoke. Lindsey (1959), however, has reported that the amount of 3,4-benzopyrene in cigarette smoke varies with the stub length, being 3·0 μg. per 100 cigarettes when the stub length is 15 mm. and 0·7 when the stub length is 35 mm. The stubs thus collect larger amounts of the cyclical hydrocarbons as smoking proceeds. It is possible that the carcinogen in cigarette condensate is something other than benzopyrene, as yet unknown. This unknown carcinogen is formed during pyrolysis as extracts of unburned tobacco or purified nicotine have been found to have no carcinogenic properties

(Gwynn and Salaman, 1954). The carcinogen is chiefly concentrated in the neutral fraction of the condensate as found by Wynder and Wright (1957).

Of the 8 malignant tumours which developed at the site of inoculation of the cigarette condensate in the lung, 6 were carcinomata and 2 sarcomata. One other malignant tumour, a squamous epithelioma, occurred in a rat inoculated with the same condensate in the experiments described in Part I where the animals were killed at intervals. Of these 9 malignant growths, 6 were found in animals which died or were killed 18 months to 2 years after inoculation. In the same period one tumour was found in the control group inoculated with eucerin, and there were 4 spontaneous pulmonary neoplasms. Bullock and Curtis (1930) and Curtis, Bullock and Dunning (1931) found 4 and possibly 5 spontaneous malignant lung tumours in a study extending over several years of a colony of 7,000 to 10,000 rats, all of which were allowed to live until death. These malignant pulmonary neoplasms were found in animals over one year old. On the other hand, Ratcliffe (1940) found no pulmonary tumours in a large colony of rats, the average age of which at death was 680 days, though in this investigation there was a bias in favour of surface tumours. Thus lung tumours in the rat, whether spontaneous or artificially produced, are associated with age. As is shown in Table II, however, of the 9 tumours in the rats inoculated with condensate, 2 occurred in animals dying up to one year and one up to 15 months after inoculation, the shortest period after inoculation being 339 days. This early occurrence of induced lung tumours as compared with those occurring spontaneously is in favour of the condensate having some carcinogenic effect. Looking at the time factor in another way the average time for cigarette condensate tumours was 533 days (76 weeks); for the one eucerin tumour, 609 days (87 weeks) and for spontaneous tumours 604 days (86 weeks). Thus the mean time for the occurrence of the condensate tumours was earlier than the mean for the control or for the spontaneous. When the time occurrence of the tumours according to histological type is considered, it is found that the carcinomata in the rats inoculated with the condensate occurred on an average of 506 days (72 weeks) and the spontaneous carcinoma at 556 days (79 weeks). The sarcomata in the animals inoculated with condensate were found on an average at 624 days (89 weeks), with eucerin, the control, 609 days (87 weeks) and the spontaneous sarcomata at 620 days (89 weeks). Thus the carcinomata arising in the rats inoculated with condensate occurred on an average 50 days earlier than the spontaneous. The sarcomata on the other hand were found at practically the same time in all groups. It is of interest to note that Wynder *et al.* (1953) observed that the mean time for the appearance of carcinomata in mice after the direct application of condensate to the skin was 71 weeks, that is almost at the same mean time as the carcinomata in the lungs of rats inoculated with condensate were observed in the present study. This time factor may in part be the explanation of the failure to produce tumours by other workers whose experiments did not extend for over 18 months.

In my 1957 experiments where the condensate from cigarette filters was used for inoculation, the carcinoma produced in the lung of a rat occurred at 506 days (72 weeks) and the sarcoma at 607 days (87 weeks). It thus seems obvious from all these results that tobacco condensate has to be in contact with the tissues of the lung for a long time before exciting a neoplastic response. Indeed as Graham *et al.* (1957) have pointed out cigarette condensate requires to be applied

to the skin of mice or of rabbits for about half the animal's life before cancer may appear.

In the human subject bronchial carcinoma occurs with increasing frequency after 45 to 50 years of age in males, reaching its maximum between 65 and 70. This is also suggestive that if cigarette smoke is a cause of lung cancer in man it must act over a long period of time, as in the experimental animal. What possibly happens is that the oily droplets of smoke, measuring 0·3 to 1 μ. in diameter, are inhaled and become deposited on the surface of the bronchial epithelium. The amount of inhaled particulate matter retained in the lungs must vary enormously depending on the condition of the bronchial mucous membrane. Ordinarily the healthy bronchial mucous membrane would, by means of ciliary action, expel most of these particles, but if the ciliated epithelium has been destroyed in places and replaced by non-ciliated, either cubical or squamous, as a result of disease or by living in a highly-polluted urban atmosphere, or as a result of habitual cigarette smoking, particles inhaled would be liable to be retained.

The ultimate fate of particles retained in the lung is of interest. As has been shown in this paper, particles of the condensate are carried away by macrophages from the site of inoculation into the lymphatics of the lung The same happens in the human subject with retained soot particles, most of which are transported in the peribronchial lymphatics towards the hilum of the lung. Any chronic peribronchial inflammation such as could arise from recurrent bronchitis would be liable to close some of these lymphatics, resulting in the arrest of the particles with the formation of focal accumulations recognisable by the naked-eye around the bronchi. If the particles have any carcinogenic power, this would be enhanced with the greater concentration. Indeed, it would be possible in the case of a smoker who inhales and who lives or had lived for a time in a highly smoke-polluted urban atmosphere to have several types of particles in the same focus of accumulation, one type exerting a co-carcinogenic effect on another with a weak carcinogenic effect. This may be the explanation of the greater incidence of bronchial carcinoma in urban areas, the retained soot particles from the polluted atmosphere acting as co-carcinogens on the particles of retained cigarette smoke, which, as has been shown in this paper, appears to be a weak carcinogen.

The higher death rate from bronchial carcinoma among United Kingdom immigrants in South Africa as reported by Dean (1959) and in New Zealand by Eastcott (1956) might be explained on the above ground. In the former country, which has the highest consumption of cigarettes in the world, most of the immigrants came from the cities in the United Kingdom and were in their twenties. As a result of climatic and polluted atmospheric conditions, together with respiratory infections due to the high-density of population in these cities, these immigrants may have suffered from repeated attacks of bronchitis at an early age, with resulting focal loss of the bronchial columnar ciliated epithelium and replacement by non-ciliated. If these subjects, after they settled in South Africa or New Zealand, were heavy smokers of cigarettes, and about this neither of these authors give detailed information, then, on account of the previous change in the bronchial epithelium, particles of inhaled cigarette condensate would be retained and exert any carcinogenic effect they might possess. It is of some significance that Dean noted in his study that many of the patients, dying of lung cancer, had suffered from recurrent bronchitis.

SUMMARY

In the first series of experiments cigarette condensate prepared in a smoking machine and suspended in eucerin when inoculated into the lung of rats, guinea-pigs and rabbits after thoracotomy caused in order of time proliferation of the basal cells of the bronchial epithelium, squamous metaplasia, followed by hyperplasia, "carcinoma *in situ*" and finally squamous epithelioma. Papillomata were also produced.

In the second series of experiments a pure strain of rats was inoculated with cigarette condensate and allowed to live until death. In these 8 (11·1 per cent) malignant lung tumours were produced whereas in the controls inoculated with eucerin alone one (2·3 per cent) malignant tumour occurred and in a group of rats not inoculated into the lungs 4 (1·4 per cent) spontaneous malignant pulmonary tumours were found. In all groups most of the neoplasms occurred in animals over 18 months old. In the period up to 15 months old, only malignant tumours associated with cigarette condensate occurred.

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THE PITUITARY CHANGES IN MICE UNDERGOING THYROID HYPERPLASIA

M. S. ISRAEL AND I. ROSEMARY ELLIS

From the Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn Fields, London, W.C.2

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IT is well recognised that any condition interfering with thyroxin synthesis leads to an increased secretion of thyrotrophic hormone from the anterior pituitary. The "thyrotrophs" undergo proliferation and may form large adenomata (Furth and Clifton, 1958). The thyrotrophic hormone in turn causes thyroid hyperplasia, provided of course the original agent interfering with thyroxin formation does not actually destroy the parenchyma of the gland.

In the following experiment mice have been treated with methylthiouracil and a low iodine diet. An attempt has been made to correlate the changes in pituitary with varying degrees of hyperplasia encountered in the thyroid.

MATERIAL AND METHODS

One hundred 4 month old C57 mice, 50 of each sex, were divided into 3 groups: a control group of 25, a group of 50 fed on methylthiouracil and a stock 41 B diet, and a group of 25 given methylthiouracil and a low iodine diet. The methylthiouracil was administered as a 0·05 per cent solution in distilled drinking water. Details of the diet have already been described (Israel and Ellis, 1960). In addition 10 mice were given the low iodine diet alone.

After 480 days the animals were killed and their thyroid glands examined, as reported previously (Israel and Ellis, 1960). The pituitary glands were removed together with a piece of underlying bone, and these were fixed either in 10 per cent formol saline or in 4 per cent formol-sublimate-saline (Swettenham, 1960). They were then dissected free from the bone, and serial paraffin sections were cut and stained as follows:

1. Erhlich's haematoxylin and eosin.
2. Mallory's trichrome stain.
3. Periodic acid Schiff (PAS) method without an orange G counterstain (Pearse, 1960).
4. Aldehyde-fuchsin stain with light green-orange G counterstain (Halmi, 1952).

RESULTS

A. *Control group*

The general pattern of pituitary cytology was uniform in the control group. Acidophil cells predominated in both sexes, and though basophils tended to be rather sparse, some glands showed considerable focal accumulations of these cells (Fig. 1). Chromophobes were more numerous than basophils.

The aldehyde-fuchsin stain revealed moderate numbers of angular, purple staining cells in the central areas of the lateral portions of the glands, while the surfaces showed none (Fig. 2 and 3). In the isthmic median parts of the glands, which connect the bulkier lateral portions, some green staining cells were present.

The PAS stain displayed cells on the inferior surfaces which stained varying shades of pink, and a few central angular cells were deeply positive, but this method was not helpful in distinguishing thyrotrophs from gonadotrophs.

It is interesting that while the formol mercury fixative brought out the aldehyde-fuchsin positive cells better than formol saline, it was the formol saline which gave the better results with the PAS stain, a finding previously noted by Swettenham (1960).

B. *Low iodine diet alone*

The changes were very slight ; the pituitaries were not enlarged, but there was a slight increase in the number of basophils. These stained deeply with PAS. More aldehyde-fuchsin positive cells were present centrally than in the controls (Fig. 4). The acidophils showed no change. The thyroids of these animals, not described previously, showed mild acinar hyperplasia without papillary ingrowth (Fig. 5).

C. *Methylthiouracil and stock diet*

There was a minimal degree of pituitary enlargement, which could be accounted for by a moderate increase in the number of basophils and a considerably augmented vascularity. Once again these basophils stained deep red with PAS in a diffuse manner. The coarse, dense, intensely-staining " T " granules described in rat thyrotrophs following thyroxin deficiency (Purves and Griesbach, 1956) were not encountered.

There was an increase in the number of aldehyde-fuchsin positive cells centrally, though some stained less strongly than others due to early degranulation (Fig. 6). Acidophils persisted and retained their granulation.

In some of the most hypercellular glands there were a few large, vacuolated " thyroidectomy " cells in the central zones, but they were too sparse to be prominent. The thyroids of such mice were particularly hyperplastic.

D. *Methylthiouracil and Low iodine diet together*

It required a combination of these two factors to produce really distinctive pituitary changes, and these were due to the appearance of many greatly enlarged " thyroidectomy " cells, as easily recognised by haematoxylin and eosin staining as by any more complicated techniques. They were polygonal and their faintly eosinophilic cytoplasm often had a festooned appearance due to intense vacuolation. The nuclei were large and had prominent nucleoli (Fig. 7). The trichrome and aldehyde-fuchsin methods gave negative results (Fig. 8). With the PAS stain some of the cells contained a few coarse, intensely-staining granules, somewhat reminiscent of the " T " granules of Purves and Griesbach, but most of the cells were PAS negative.

These cells were constantly present in large numbers, being especially abundant centrally (Fig. 9), and to a lesser extent peripherally. In 19 of the 25 pituitaries they were aggregated into discrete adenomata, though in 11 these were of micro-

scopical size only. Such micro-adenomata were usually central (Fig. 10), though sometimes peripheral also (Fig. 11), and elsewhere there was a copious infiltration of similar cells.

In 8 mice the adenomata was so large that the pituitaries were macroscopically massive (Fig. 12). The cells of these adenomata were smaller, more spherical, less vacuolated and more basophilic than in the smaller collections, and they were arranged in solid clusters surrounded by a highly vascular stroma (Fig. 13). In the engorged sinusoids there were lining cells filled with haemosiderin pigment (Fig. 14), perhaps derived from previous haemorrhages in engorged glands. In some of the larger tumours mitotic figures were quite frequent (Fig. 15), but in none was there evidence of local invasion or distant metastasis. In even the largest tumours the remains of compressed parenchyma was present, and in it fully granulated acidophils were still to be found, but aldehyde-fuchsin positive cells were absent.

In all the mice there was profound thyroid hyperplasia, but there was no absolute correlation between pituitary size and thyroid proliferation. In only 4 mice with thyroid adenomata were there also pituitary adenomata, though 3 others had micro-adenomata in their pituitaries. In the remainder there was merely a diffuse infiltration of thyroidectomy cells throughout the parenchyma.

Conversely, only 4 of the 8 large pituitary adenomata were associated with thyroid adenomata.

There was no special sex incidence of pituitary adenomata.

One mouse died spontaneously of a metastasising thyroid cancer. Unfortunately the pituitary was too autolysed for adequate histological appraisal, but it did not appear to be conspicuously enlarged.

DISCUSSION

With the development of histochemical techniques over the last decade, much progress has been made in elucidating the nature of the pituitary basophils. Pearse (1952) in particular has demonstrated the importance of the PAS stain in identifying the mucoprotein gonadotrophic and thyrothrophic hormones present in basophils and also in some chromophobes, while Halmi (1952) has employed the aldehyde-fuchsin technique to distinguish between different types of basophils. Purves and Griesbach (1951*a*) have applied these methods extensively to the rat pituitary and have been able to divide the basophils into two main groups : a central group, consisting of cells staining purple with aldehyde-fuchsin (" beta cells ") and identified as thyrotrophs, and a peripheral group, remaining unstained with aldehyde-fuchsin and taking up the light green counterstain (" delta cells "). These are believed to be gonadotrophs.

By contrast the mouse pituitary has been far less exhaustively studied. Halmi and Gude (1954) described a picture similar to that of the rat pituitary, with centrally placed aldehyde-fuchsin positive cells, and negatively staining cells near the area of the isthmus. The histological appearances of the control group described above conform to Halmi and Gude's description quite closely, though it is evident that much has still to be learnt about the cytology of the normal mouse pituitary.

The comparatively minor changes induced in the mouse pituitary by methylthiouracil alone are interesting when the great thyroid hyperplasia is remembered.

Clausen (1956) fed rats on thiouracil and also noted moderate basophil hyperplasia after one year, most of the cells being aldehyde-fuchsin positive. By 18 months many of the basophils were degranuated, yet thyroidectomy cells were very scanty. Only after 2 years were small micro-adenomata to be found. Thyroxin deficiency in rats, whether due to thyroidectomy (Knigge, 1958) or following thiouracil administration (Sellars, Hill and Lee, 1953), produces a rapid degranulation of acidophils. This does not occur in mice (Halmi and Gude, 1954), a feature the mouse pituitary has in common with that of the hamster (Serber, 1958).

It required a combination of low iodine diet and methylthiouracil to produce the very conspicuous " thyroidectomy cell " in large numbers, sometimes to the extent of forming large tumours almost completely obliterating the normal parenchyma. It is noteworthy that these cells are recognised quite as easily by haematoxylin and eosin staining as by any histochemical methods, as they are PAS and aldehyde-fuchsin negative.

The nature of these cells has long given rise to speculation because of their indeterminate staining with trichrome methods and their obvious morphological dissimilarity to the common chromophobe. Halmi and Gude (1954) produced adenomata composed of these cells by subjecting mice to radiothyroidectomy, and Burt, Landing and Sommers (1954) administered radioactive iodine to the same effect. These latter authors described the cells in great detail, and noting their ambivalent staining reactions with commonly used techniques, called them

EXPLANATION OF PLATES

FIG. 1.—Normal mouse pituitary. Both acidophils and basophils are shown, but the basophils stain much more darkly, and are rather numerous in this field. Mallory's trichrome. ×165.

FIG. 2.—Normal mouse pituitary. A lateral portion of the anterior lobe showing the presence of darkly-staining cells in the central area only. Halmi aldehyde-fuschin. ×75.

FIG. 3.—Normal mouse pituitary. A detailed view of the central area of Fig. 2 showing the arrangement of positively-staining cells. Halmi aldehyde-fuchsin. ×300.

FIG. 4.—Pituitary of mouse fed on low iodine diet. Some increase in number of positively-staining cells in the central part. Halmi aldehyde-fuchsin. ×165.

FIG. 5.—Thyroid of mouse fed on low iodine diet. There is mild acinar hyperplasia only. Haematoxylin and eosin. ×112.

FIG. 6.—Pituitary of mouse given methylthiouracil. An increased number of central positively-staining cells, some of which show evidence of early degranulation. Halmi aldehyde-fuchsin. ×165.

FIG. 7.—Pituitary of mouse fed on methylthiouracil and low iodine diet. Typical " thyroidectomy " cells in a central cluster. Haematoxylin and eosin. ×165.

FIG. 8.—Pituitary of mouse fed on methylthiouracil and low iodine diet. The thyroidectomy cells do not stain with aldehyde-fuchsin, but a few remaining small cells stain positively. Halmi aldehyde-fuchsin. ×165.

FIG. 9.—A diffuse central infiltration of thyroidectomy cells and a small micro-adenoma peripherally. Mallory's trichrome. ×82.

FIG. 10.—Two central micro-adenomata. Mallory's trichrome. ×82.

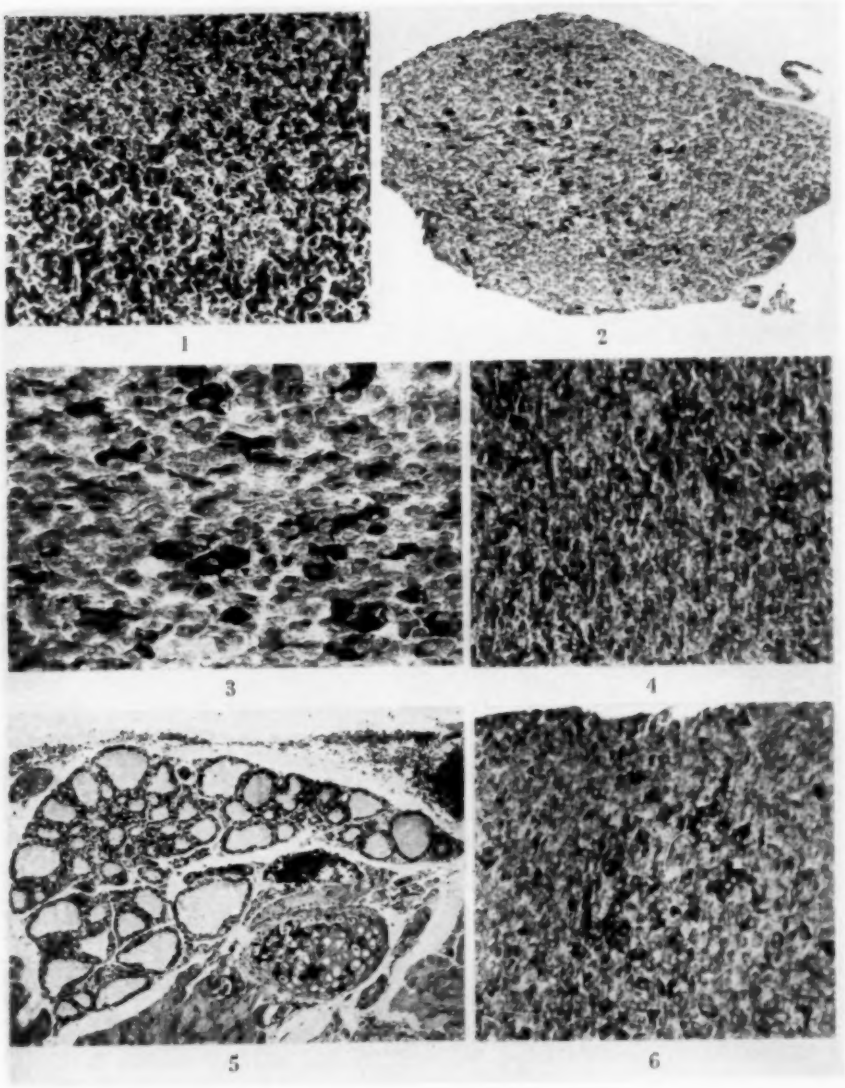
FIG. 11.—A large peripheral microadenoma. The vacuolation of the cells is evident. Mallory's trichrome. ×165.

FIG. 12.—A large adenoma. Haematoxylin and eosin. ×82.

FIG. 13.—The arrangement of adenoma cells in solid clumps is seen. The cells are quite vacuolated. A mitotic figure is present. Haematoxylin and eosin. ×410.

FIG. 14.—Many of the sinus-lining cells of the adenoma contain iron pigment. Perls's stain with neutral red counterstain. ×205.

FIG. 15.—An exuberant adenoma with mitotic figures. Haematoxylin and eosin. ×205.



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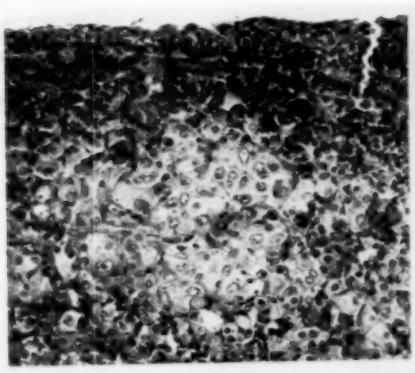

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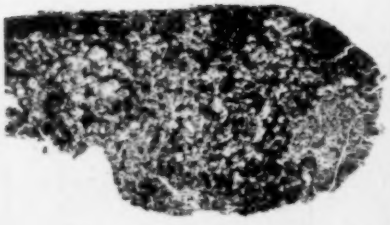

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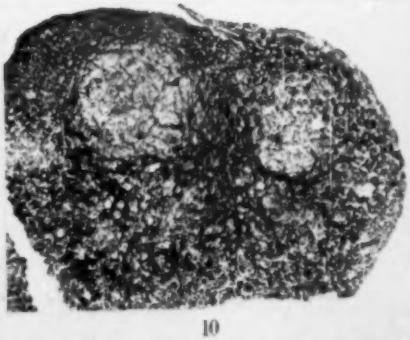

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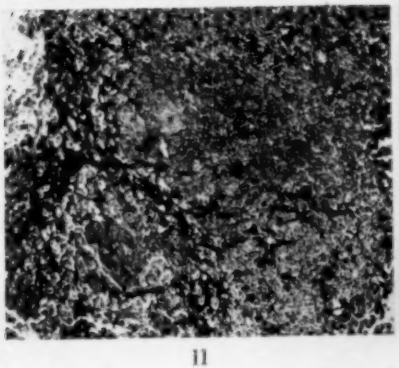

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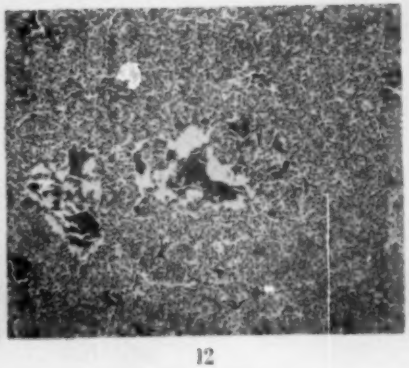

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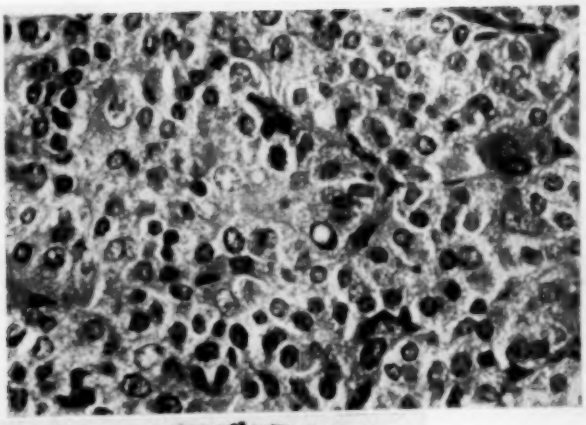

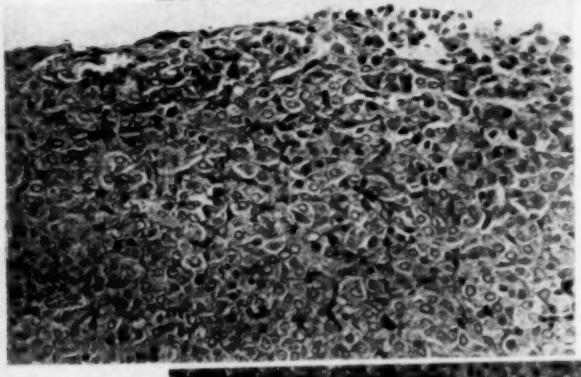

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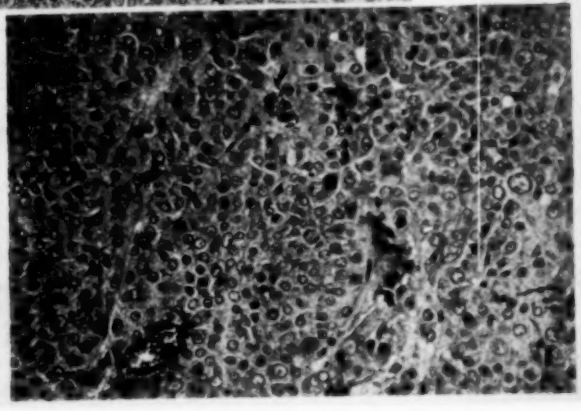

" amphophils ". These cells are also found in the rat (Purves and Griesbach, 1956), hamster (Serber, 1958), and human pituitary (Russfield, 1955) in states of reduced thyroxin synthesis produced experimentally or arising secondary to disease.

Their origin is complicated by the fact that after gonadectomy large cells are once again seen in the pituitary. Griesbach and Purves (1960) have reported basophil adenomata in the pituitaries of rats of both sexes after gonadectomy. These tumours were of " delta cells ", but often it is impossible to decide their origin by aldehyde-fuchsin staining (Swettenham, 1960, personal communication). Indeed all too often these techniques fail just when they are most urgently needed. At present it seems that the only reliable guide to the origin of " amphophils " lies in ascertaining the cause of their appearance. In the present experiment they manifestly followed interference with thyroxin synthesis, and both Purves and Griesbach (1956) and Elftman (1958) have provided good evidence for the transformation of degranulated thyrotrophs into typical thyroidectomy cells. Purves and Griesbach (1951*b*) suggested that the granules in the thyrotrophs were stored hormone, and that any stimulus to increase its formation would be accompanied by its disappearance from the cells.

It is easy to correlate a pattern of intense thyroid hyperplasia with the extent of thyroidectomy cell infiltration of the pituitary, but no definite relationship could be made between the presence of large pituitary tumours and thyroid adenomata. The extreme difficulty in producing malignant change in these thyroids has already been emphasised (Israel and Ellis, 1960), and it is significant that in the one animal which had an indisputable thyroid carcinoma the pituitary gland was not greatly enlarged.

Functional thyrotrophic adenomata have been extensively studied. Haran-Ghera, Pullar and Furth (1960) have investigated the neoplastic potentiality of thyroid hyperplasia induced by such an adenoma, using grafts on to other mice. Hormone dependency remained for 4 generations and there was no irrefutable evidence of malignancy. On the other hand less work has been done in correlating the pituitary changes with the degree of thyroid hyperplasia which follows the use of thiouracil derivatives in animals.

Moore, Brackney and Bock (1953) fed 4 mice on thiouracil, and on killing them 534 days later noted definite evidence of thyroid malignancy in 2 and questionable evidence in the other 2, yet it was these last 2 mice that had large pituitary adenomata. In one of the first 2, however, there were only micro-adenomata in a moderately enlarged gland and in the other gland was not even greatly enlarged and was merely infiltrated with " chromophobe " cells. Sellars, Hills and Lee (1953) also noted great thyroid hyperplasia in rats treated with propylthiouracil and dried thyroid, and these animals showed many pituitary adenomata which were described as " chromophobe ".

It would appear that only prolonged, severe interference with thyroxin synthesis can stimulate the production of thyroidectomy cells in mice, and that considerable thyroid hyperplasia may occur with only slight pituitary changes. Once, however, large numbers of thyroidectomy cells are present there is a tendency for compact thyroid adenomata to appear. The rarity of malignant change suggests that these cells are not related to thyroid carcinoma. The supervention of cancer presumably requires another factor in addition to thyrotrophic hormone.

SUMMARY

A group of C57 mice were subjected to varying degrees of interference with thyroxin synthesis, and after 480 days a correlation between pituitary and thyroid changes was made.

A low iodine diet alone produced a slight increase in aldehyde-fuchsin positive cells of the pituitary, while the thyroids showed mild acinar hyperplasia.

Methylthiouracil alone caused a rather greater increase in the number of aldehyde-fuchsin positive cells, though some of these showed evidence of early degranulation. The thyroids showed great papillary hyperplasia.

A combination of low iodine diet and methylthiouracil produced a pronounced infiltration of the pituitary with thyroidectomy cells often arranged in adenomata. There were sometimes thyroid adenomata also, but there was no direct correlation between these and the pituitary adenomata.

It is concluded that although the most intense thyroid hyperplasia is invariably associated with thyroidectomy cell infiltration of the pituitary, these cells are not directly related to thyroid cancer which is rare. At the most the hormone they secrete may potentiate a primary neoplastic stimulus.

We are grateful to Professor G. J. Cunningham for his interest and advice: to Mr. K. Swettenham of the Bernhard Baron Institute of Pathology, the London Hospital, for his assistance with staining techniques, and to Mr. A. L. E. Barron for the photomicrographs.

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RETICULAR TUMOURS IN MICE SUBJECTED TO PROLONGED ANTIGENIC STIMULATION

D. METCALF

From the Cancer Research Laboratory, Walter and Eliza Hall Institute, Royal Melbourne Hospital Post Office, Australia

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It has frequently been suggested that prolonged hyperplasia in a tissue may increase the incidence of subsequent neoplasia in that tissue. Experimental evidence supporting such a concept has been accumulated for a number of tissues under endocrine regulation—pituitary, thyroid, ovary, testis, adrenal and breast (see review by Furth, 1953). In other tissues, the association between hyperplasia and subsequent neoplasia is less certain, primarily because little is known of factors capable of inducing sustained hyperplasia in these tissues.

It is well known that the injection of foreign antigenic material into a wide variety of animals is followed, at least for a time, by hyperplasia of the reticular tissues—the response mainly involving cells of the lymphocyte, plasma cell and reticulo-endothelial series.

It is apparent from the studies on the reticular tissues of animals maintained in a germ-free state (Gordon, 1959) that the "normal" animal is, in fact, not normal but one in which there has been a hyperplastic response on the part of the reticular tissues to stimulation by bacterial and other foreign antigens encountered in everyday life. It is possible that the continuing stimulation of these tissues by such antigens may, in part, be responsible for the occurrence of some of the reticular tumours in both humans and animals.

In the present experiments an attempt has been made to test this hypothesis by subjecting mice to prolonged antigenic stimulation throughout life, and observing the effects of this stimulation on the incidence of various reticular tumours in these mice.

MATERIAL AND METHODS

Mice.—Mice used were 5–7 weeks old males and virgin females of the inbred strain C3H, maintained in this institute. The mice were housed in groups of five, in metal boxes, with sawdust bedding. They were fed Barastoc dog pellets and water *ad libitum*, with carrots twice weekly and supplemental greens. The room temperature was maintained at 75° F.

Antigens.—Salmonella adelaide flagellar antigen was prepared according to the method described by Nossal (1959*a*). The suspension was diluted 1 : 100 in normal saline, the injected dose per mouse being 0·2 ml.

Bovine Serum Albumin (BSA) (Fraction V. Abbott) was dissolved in normal saline. The dose per mouse was 4·0 mg., in 0·2 ml. saline.

Injection procedure.—Each mouse was injected, at weekly intervals with either Salmonella antigen or BSA. Control mice were injected with saline. Injections

were made intraperitoneally, using tuberculin syringes and No. 27 needles. Each mouse was injected with an individual sterile syringe and needle to prevent accidental transplantation of neoplastic cells from one mouse to another during the repeated injections. Syringes were washed thoroughly after use, then autoclaved. Four hundred syringes and nee"es were used repeatedly at weekly intervals for these injections. The control, saline-injected, mice were examined serologically at regular intervals for the presence of Salmonella and BSA antibodies, but the sera of these mice remained free of detectable antibodies throughout life. This indicated that no significant contamination of syringes occurred during repeated use.

Pathology.—Mice were killed when moribund, and the reticular tissues fixed in 10 per cent Zenker's formalin. The tissues were dehydrated, and blocked in paraffin. They were sectioned at 7 μ. and stained with haematoxylin and eosin.

Tumour transplantations.—Neoplastic tissue for transplantation (usually spleen and/or involved lymph nodes) was removed aseptically, minced in chilled sterile normal saline, and injected intramuscularly into the thigh muscles of four to six, 6 weeks old C3H mice of the same sex. Half the recipients were injected at weekly intervals with the relevant antigen, the other half with saline. Recipient mice were observed for a period of 6 months, and if still alive at this time, killed and autopsied.

Antibody titrations.—At intervals throughout life, the sera of mice receiving antigen injections were examined for the presence of antibodies.

Salmonella flagellar antibodies were titrated by the immobilisation technique described by Nossal (1959*b*).

BSA antibodies were titrated, using Fisher's modification of the Boyden tanned sheep red cell technique (Fisher, 1952).

Paper electrophoresis.—Sera for examination electrophoretically, were placed on strips of Whatman No. 1 filter paper, soaked in veronal buffer (pH 8·6 ; ionic strength 0·075). The voltage applied was 2–3 volts/cm. After 16 hours at 25° C., the strips were fixed by heating to 120° C. and stained with bromphenol blue.

RESULTS

Two types of observations were made on the antigen- and saline-injected mice. In the first, mice of all three groups were killed at various intervals after injections were commenced, and their reticular tissues weighed, and examined histologically. The number of mice so examined was : *Saline injected*, 40, *Salmonella antigen injected*, 41 and *BSA injected*, 45. Observations were made on groups of these mice at the following times after commencement of injections—4, 8, 14, 18, 35, 50 and 64 weeks.

In the second series of experiments, run simultaneously, groups of mice injected with saline and the two antigens were observed until moribund. At intervals, estimations were made on these mice of white cell levels and antibody levels in the tail blood. When moribund, the mice were killed and examined histologically. The number of mice followed in this manner was : *Saline injected* 120(52F, 68M), *Salmonella antigen injected* 119(57F, 62M), *BSA injected* 135 (71F, 64M).

General effects of antigenic stimulation

The mean life span of the antigen-injected mice was shortened (saline group 22·1 months; Salmonella antigen group 18·4 months, and BSA group 20·4 months).

In the mice injected with Salmonella antigen, in which this shortening of life span was most evident, the primary cause of early deaths was the development of severe amyloid disease, the first deaths occurring at 14 months. The organs most severely involved were the spleen, kidney and liver. Amyloid disease was the chief cause of death in 78 per cent of the mice of this group.

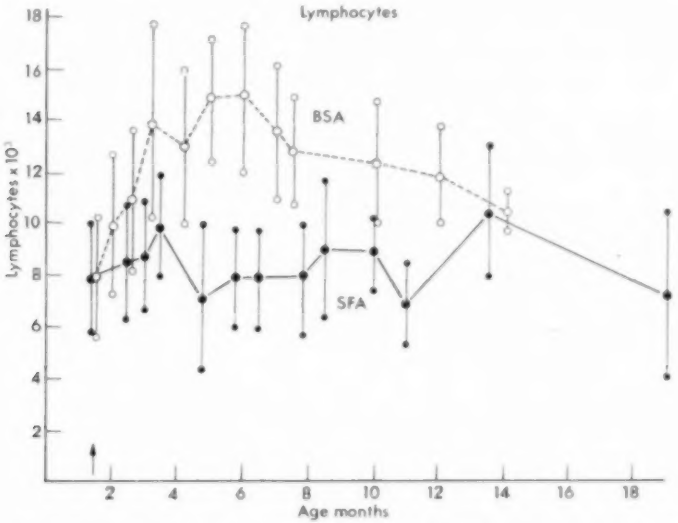

FIG. 1.—Lymphocyte levels in mice injected with either bovine serum albumin (BSA) or Salmonella flagellar antigen (SFA). Each point represents the mean and standard deviation of levels in ten mice. Arrow indicates commencement of injections.

Amyloid disease was much less common, and of a much milder degree in the mice injected with BSA. In this group, the first death from amyloid disease did not occur until 21 months, and the final incidence of the disease was only 23 per cent—most of the lesions being minimal.

In the saline-injected control group the incidence of amyloid disease was 8 per cent, most cases being associated with the presence of chronic abscesses in the animals.

General effects of antigenic stimulation on reticular tissues

White cell counts were performed on the tail blood of ten mice in each of the three groups at monthly intervals throughout life.

Mice injected with BSA, developed a slight lymphocytosis, one month after injections were commenced, which persisted for 12 months (Fig 1). After this time lymphocyte levels returned to control values. At its maximum, the lympho-

cytosis represented an elevation of lymphocyte levels to 50–100 per cent above those in the control mice. No changes from normal occurred in the lymphocyte levels of mice injected with Salmonella antigen. In both experimental groups, after the age of 12 months, polymorphonuclear levels rose progressively, reaching levels of three times those of control mice (Fig. 2). Mice with amyloid disease tended to have higher polymorph levels than others.

No classical plasma cells were observed in the peripheral blood of antigen-injected mice at any stage during the experiments. However, lymphocytes with basophilic cytoplasm (Downey Types I and II) were common in these mice.

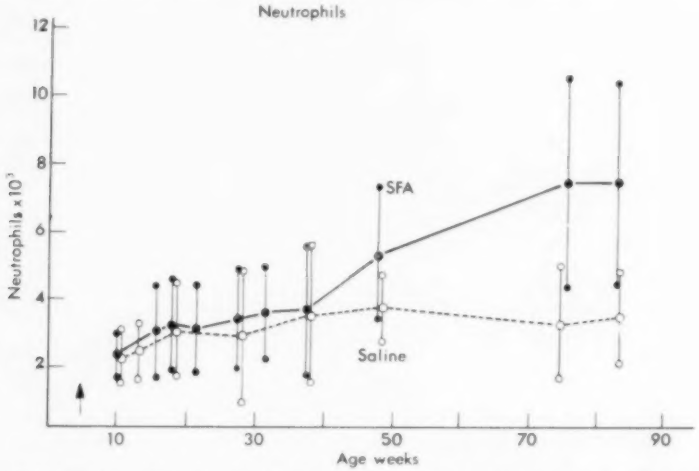

FIG. 2.—Polymorphonuclear neutrophil levels in mice injected with Salmonella flagellar antigen (SFA) compared with those in saline-injected control mice. Each point represents the mean and standard deviation of levels in ten mice. Arrow indicates commencement of injections.

Eosinophil levels showed no consistent alterations in antigen-injected mice. However, no special measures were taken to observe the mice during basal activity.

In the mice killed at various intervals after the commencement of antigen injections, little of significance was observed when the various reticular organs were weighed. No differences were found in the weights of the lymph nodes or thymus between the experimental groups and the control mice at any of the ages examined. Antigen-injected mice showed slight splenomegaly (weights up to 50 per cent above those of saline-injected mice). After 35 weeks even this difference was not seen.

Histologically, all elements in the lymphoid tissues of the antigen-injected mice were hyperplastic during the first year of the injections. Although germinal centres were enlarged, and showed increased mitotic activity, they never reached the size seen in other species stimulated by foreign antigens. Plasma cell accumulations were prominent in the medullary regions of the lymph nodes, and in the splenic red pulp, but this response was maximal by twelve months. Giant cells also accumulated in the spleens of antigen-injected mice during the first

year. Small accumulations of mature lymphocytes and plasma cells were also common around major blood vessels in the kidneys of antigen-injected mice.

The general impression gained from the mice sampled was that there was an initial hyperplastic response, which became maximal by 12 months of age and decreased after this time.

Antibody titrations

Serum antibody levels were not exhaustively followed in all the mice involved in the long term experiments. Observations were made at irregular intervals throughout the duration of the experiments. Antibody titres were also determined on some of the sera collected at the time of killing the antigen-treated mice.

In both groups, high levels of antibodies (to 2×10^6 units for Salmonella antigen and $0{\cdot}5 \times 10^6$ for BSA) were present in most mice by the end of the first year. After this time, antibody levels in individual mice became more variable and fell an average of one log. At no time did antibodies disappear from the sera of antigen-injected mice. There was no evidence of the development of immunological paralysis even in the BSA group, which were being injected with relatively large doses of foreign protein.

Mice, terminally ill, especially those with amyloid disease or reticular neoplasms, tended to have low antibody levels.

In no instance did mice with reticular tumours have exceptionally high antibody titres. This suggested that none of these tumours were composed of cells capable of producing complete antibody.

Incidence of Reticular tumours

Reticular tumours were classified into one or other of six types—thymic lymphoma, non-thymic lymphoid leukaemia, reticulum cell sarcoma, atypical reticulum cell sarcoma, plasma cell tumour and myeloid leukaemia. In general the criteria for classification were those laid down by Dunn (1954). However certain points need comment. The diagnosis of reticulum cell sarcoma was confined to those tumours in which there were uniform sheets of either spindle-shaped or polygonal reticulum cells, with a minimum of other cells types present. The atypical reticulum cell sarcomata included the reticulum cell sarcomata Types B and C of Dunn. These were tumours in which there was a mixture of cell types present—usually lymphocytes, reticulum cells, plasma cells, eosinophils and polymorphs, with binucleate, or occasionally multinucleate, giant cells. Characteristically, this latter lesion caused massive enlargement of the spleen and mesenteric node, with or without involvement of other lymph nodes. Invasion of the liver and kidney, although present, was usually minimal.

The plasma cell tumours encountered were almost all localised in the mesenteric area and were similar to those described recently by Potter and Robertson (1960).

In Table I is presented a summary of the incidence of reticular neoplasms in the two antigen-treated groups and the saline-injected control mice.

The incidence of reticular tumours was low in the control mice during the first year (2 per cent). However, during the second year, a variety of neoplasms occurred, giving a final total incidence of 12 per cent. Four cases of reticulum cell sarcoma occurred, all in female mice. In addition, a further 16 cases (14 per cent) of atypical reticulum cell sarcoma occurred. Data will be presented

TABLE I.—*Incidence of Reticular Tumours in C3H Mice Subjected to Repeated Antigenic Stimulation*

Antigen	Number mice	Mean age at death (months)	Thymic lymphoma (%)	Non-thymic lymphoid leukaemia (%)	Reticulum cell sarcoma (%)	Plasma cell tumour (%)	Myeloid leukaemia (%)	Total per cent incidence of reticular tumours	Atypical reticulum cell sarcoma (%)	Hepatoma (%)	Lung tumour (%)	Other tumours†
Saline	120	22·1	2(11·3*)	6(21·4)	3(23·5)	0	1(13·0)	12	14(21·0)	33(23·0)	33(24·7)	4 O.T. 7 B.T. 1 Osteoma
Salmonella flagellar antigen	119	18·4	4(10·8)	10(18·1)	2(21·0)	2(18·0)	1(17·0)	19	4(22·4)	29(18·7)	16(20·1)	9 O.T. 5 B.T.
Bovine serum albumin	135	20·4	5(6·7)	11(20·6)	9(22·0)	6(21·6)	0	31	12(20·5)	24(21·5)	31(23·9)	6 O.T. 5 B.T.

* Mean age at death in months.
† O.T. = Ovarian tumour.
B.T. = Breast tumour.

later, suggesting that this latter lesion may not be neoplastic, but rather granulomatous in nature, and these lesions have accordingly been listed as a separate category.

In the mice injected with Salmonella flagellar antigen, a final incidence of reticular tumours of 19 per cent was observed. This included two cases of plasma cell tumour, one located in the mesentery, one originating in a Peyer's patch. In addition, five cases (4 per cent) of atypical reticulum cell sarcoma were seen. In both saline and Salmonella groups a single case of myeloid leukaemia occurred.

In the mice injected with bovine serum albumin, an incidence of 31 per cent reticular tumours occurred. These comprised 5 per cent thymic lymphoma, 11 per cent non-thymic lymphoid leukaemia, 9 per cent reticulum cell sarcoma and 6 per cent plasma cell tumour. There was some shortening of the latent period of the thymic lymphomata in this group, but the average latent periods for the other neoplasma were similar to those of the control mice.

The lymphoid neoplasms were divided equally between male and female mice, but 10 of the 12 reticulum cell sarcomata and all if the 8 plasma cell tumours occurred in female mice. In addition, 12 per cent of the BSA-injected mice developed atypical reticulum cell sarcomata.

In one instance, plasma cell tumours occurred in two mice in a single cage, but these were separated by a time interval of 7 months. In the mice with plasma cell tumours the distribution of neoplastic plasma cells was similar in all cases. The primary location was in the mesenteric region, small neoplastic deposits being seeded through the fat of the mesentery. The mesenteric lymph node was involved in all but one case, which appeared to have originated in a Peyer's patch on the ileum. Neoplastic cells were located mainly in the medullary region of affected lymph nodes, but as the disease progressed these spread to involve the whole organ. Neoplastic cells were common in the kidney and the perirenal lymph nodes, but rare in the liver and spleen. In three cases, a moderate amount of ascitic fluid was present. This contained large numbers of neoplastic cells with smaller numbers of lymphocytes and desquamated peritoneal cells.

The sera from five of the cases of plasma cell tumour were examined by paper electrophoresis. Four of the five sera showed large peaks of the b-globulin region. The fifth serum showed no distinct change. The sera of mice transplanted with two of the above plasma cell tumours were also examined, and showed b-globulin peaks similar to those in the original animals.

The incidence of neoplasms of other tissues was incidentally observed at autopsy in all three groups. The incidence of lung tumours in mice injected with Salmonella antigen, and the incidence of hepatomata in BSA-injected mice were lower than in control mice. The incidence of ovarian and breast tumours was approximately equal in all groups

Results of transplantation of tumours of antigen-treated and normal recipients

Many of the reticular neoplasms occurring in both antigen-treated groups were tested by transplantation to four, 6 weeks old C3H mice of the same sex. After transplantation, two of the recipients were injected weekly with the relevant antigen, two with saline. The recipient mice were observed for a period of 6 months after transplantation.

TABLE II.—*Results of Transplantation of Reticular Tumours Arising in Antigen-treated Mice*

Antigen	Thymic lymphoma*	Non-thymic lymphoid leukaemia	Reticulum cell sarcoma	Plasma cell tumour	Atypical reticulum cell sarcoma	Myeloid leukaemia
Salmonella flagellar antigen	3/3	6/9	1/1	—	0/2	1/1
Bovine serum albumin	6/6	6/11	6/7	2/5	1/10	—

* Number of tumours which proved transplantable/number tested.

Table II shows the results of transplantation of the various types of reticular tumours. In the majority of instances, the recipients developed thigh tumours, with or without generalised spread of the disease, within 2 months of transplantation. In the case of the reticulum cell sarcomata, positive transplants were slower to develop, the average latent period being 4–6 months.

The thymic lymphomata and reticulum cell sarcomata proved readily transplantable, whilst approximately half of the non-thymic lymphoid leukaemias and plasma cell tumours were successfully transplanted. A striking feature was the failure of the lesions diagnosed histologically as atypical reticulum cell sarcomata to grow progressively after transplantation to either antigen-injected or saline-injected mice. In each case the typing of the original tumour was made before the results of transplantation were known. In only one instance did the morphology of the transplanted tumour conflict with the diagnosis of the original tumour. This was the single atypical reticulum cell sarcoma which grew on transplantation, and the transplanted tumour was composed wholly of neoplastic lymphoid cells.

No unquestionable examples of an antigen-conditioned tumour was found during these transplantations. (A conditioned tumour would be one arising in an antigen-treated mouse, and only growing progressively on transplantation in antigen-treated, but not saline-injected, mice.).

However, several observations were made which suggested the possible occurrence of partially conditioned reticular tumours in the antigen-treated mice. One plasma cell tumour, arising in a BSA-treated animal, was passaged three times in BSA-treated recipients before transplanted tumours became detectable in Passage 1 and 2 recipient mice injected with saline. During Passage 3, however, the tumour grew equally rapidly in BSA- and saline-injected mice. The cells of this plasma cell tumour were unusually regular in morphology, and exhibited well-developed differentiation towards mature plasma cells.

A second plasma cell tumour, arising in a BSA-treated animal, produced transplanted tumours in both BSA-injected recipients, but not in the saline-injected mice. This tumour was not passaged further.

The only reticulum cell sarcoma, arising in BSA-treated mice, which failed to grow on transplantation was tested only in normal mice, no BSA-injected mice being included in the recipients.

In all the other transplantations shown in Table II, the tumours tested grew equally rapidly in both antigen- and saline-injected recipients.

DISCUSSION

Previous reports have been made of the induction of leukaemia, or leukaemoid states, in animals by the injection of foreign proteins. Pentimalli (1954) claimed to have induced monocytic leukaemia in rabbits by long-term injections of milk and other antigenic substances. Oliver and Katzman (1938) reported the development in mice of a leukaemoid state following repeated injections of casein. However, in neither case was positive evidence presented for the neoplastic nature of the lesions resulting from the protein injections.

The present results suggest that prolonged injection of foreign protein caused a moderate increase in the incidence of reticular tumours in the injected mice.

In some mice at autopsy it was difficult to determine whether the changes in the reticular tissues were hyperplastic or were showing early neoplastic changes. Although these lesions were not usually transplantable, they gave the impression of being in an intermediate state between hyperplasia and neoplasia.

The apparently negative results in mice injected with Salmonella flagellar antigen are difficult to interpret since the life span of the mice was shortened by the experimental procedure. In this regard it may be significant that there was an increased incidence of thymic and non-thymic lymphoid tumours—this group comprising the only reticular tumours with an average latent period shorter than the mean life span of this group of mice. The lower incidence of atypical reticulum cell sarcoma in this group may also be due to the fact that most of this group were dead before the average age of occurrence of this lesion.

In the mice injected with bovine serum albumin, in which there was no significant shortening of the life span, the incidence of all types of reticular neoplasms was approximately doubled. By contrast, there was no rise in the incidence of myeloid leukemia or atypical reticulum cell sarcomata. The neoplastic nature of this latter lesion must seriously be questioned in view of the failure of these " tumours " to grow progressively, after transplantation to either antigen-treated or normal isologous recipients. It is possible that some may have been very slowly growing neoplasms, and that the observation period of 6 months was too short to allow detectable growth to take place. However, the histological appearance of these lesions frequently suggested a granulomatous or chronic inflammatory response, and such may have been their true nature.

The occurrence of eight cases of plasma cell tumours in the BSA-injected mice, and of two in the Salmonella antigen-injected group, is of considerable interest. Although the absolute numbers are small, the spontaneous incidence of plasma cell tumours in our C3H substrain is very low, since none occurred in the control mice, and no such tumours have been seen in several hundred autopsies on C3H mice aged between 1 and 2 years. Further, the mesenteric location of the tumours suggested a relationship with the injected antigen.

Plasma cell tumour development has been reported in BALB/c mice by Merwin and Algire (1959) after the implantation of millipore diffusion chambers, containing C3H tissue. Potter and Robertson (1960) described the occurrence of seven plasma cell tumours in a small group of female BALB/c mice injected intraperitoneally with paraffin oil adjuvant mixed with heat killed staphylococci.

These findings in association with the present results suggest that prolonged antigenic stimulation may be an important aetiological factor in plasma cell tumour development in mice.

In the earlier reports, and in the present series, the plasma cell tumours occurred in female mice. The reticulum cell sarcomata were also more numerous in the female mice. This suggests that sex hormones may be involved in the aetiology of these tumours in mice.

The results of transplantation of the reticular tumours occurring in antigen-stimulated mice, both into antigen-treated and normal mice, gave little support to the possibility that the progressive growth of these tumours was dependent on the continued presence of the antigen. There were some suggestions that two of the plasma cell tumours grew better in antigen-treated mice, but this effect was a transitory one. However, such negative findings on the growth requirements of established reticular tumours, are not necessarily relevant to the question of whether or not antigenic stimulation was a determinant in the genesis of these same tumours.

In experiments involving repeated injection of inbred mice, possibly already leukaemic, there is a real risk of accidental transplantation of viable leukaemic cells from one animal to another. In the present experiments care was taken to avoid such a possibility, and an analysis of the incidence and time of occurrence of reticular tumours in the various cages gave no indication that accidental transplantation of leukaemic cells had occurred.

The present findings raise the possibility that stimulation of the reticular tissues by foreign antigens, possibly coupled with individual idiosyncrasy in responsiveness to such stimulation, may be an aetiological factor in human reticular tumours. Pertinent to this question is perhaps the fact that acute lymphoid leukaemia is common in childhood, and occurs in an age group in which lymphoid hyperplasia, presumably a response to antigenic stimulation, is almost universal. In an epidemiological survey on aetiological factors in child leukaemia, Manning and Carrol (1957) reported that there was a higher incidence of allergy—hay fever, asthma and hives—in leukaemic children and their mothers than in control children and mothers.

The mechanism by which the increased reticular tumour incidence was produced by antigenic stimulation is not known. The increased mitotic activity of the reticular cells during the hyperplastic response may have allowed more opportunities for random neoplastic mutations. Alternatively, the stress placed on the reticular tissues may have allowed the activation of latent tumour viruses in these mice.

SUMMARY

C3H mice were injected intraperitoneally, at weekly intervals for life, with either saline, Salmonella flagellar antigen or bovine serum albumin.

The incidence of reticular tumours in these mice was: saline-injected 12 per cent, Salmonella antigen-injected 19 per cent and BSA-injected 31 per cent. Six per cent plasma cell tumours occurred in the BSA-injected mice, and 2 per cent in the Salmonella antigen-injected mice, but none in the control mice.

The age of occurrence of the reticular tumours in antigen-treated mice was similar to that in control mice. None of the reticular tumours was dependent for its progressive growth, after transplantation, on the continued presence of the relevant antigen.

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EPITHELIAL TUMOURS OF THE SKIN OF SHEEP

Tumours of Areas Exposed to Solar Radiation

L. C. LLOYD

From the Department of Veterinary Pathology and Bacteriology, University of Sydney, Australia

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Neoplastic conditions of the skin in various species have been attributed to the action of sunlight, particularly its ultra-violet portion.

The occurrence of basal cell and squamous cell epitheliomata on exposed parts of the body in white skinned man living in latitudes with intense, prolonged, solar radiation has been the subject of considerable study, (Paul, 1918; Molesworth, 1927, 1944; Blum, 1945, 1955, 1959; Mackie and McGovern, 1958; Cooper, 1959; Belisario, 1959). There appears little doubt that sunlight is an aetiological factor in some skin cancers of man.

Epithelial tumours of the skin, analogous to those in man, occur in cattle, sheep, goats and cats. In most instances predilection sites for such neoplasms are areas poorly covered by hair or wool and lacking pigmentation, and it seems that sunlight could be an important factor in their causation.

In cattle, tumours commonly develop in and around the eye (Monlux, Anderson and Davis, 1956; Russell, Wynne and Loquvan, 1956). Some arise on the eyeball and others on the eyelids. Sunlight may be concerned in the aetiology of both types, but the evidence is more definite in regard to those on the eyelids. The work of Guilbert *et al.* (1948), Anderson, Lush and Chambers (1957) and French (1959) indicates that cattle lacking pigmentation in the eyelids are more prone to the condition than those with pigment. In addition, if cattle are selected for breeding on the basis of a high degree of pigmentation of the eyelids, the incidence of these tumours in their progeny will be reduced (Anderson and Chambers, 1957).

In regard to epithelial neoplasms in other sites on the bovine, Drabble (1929) noted that of the 159 tumours he observed 72 per cent were on white cattle or on the white areas of coloured cattle.

White skinned goats are subject to squamous cell epitheliomata of the skin. Thomas (1929) gave a detailed account of tumours occurring in Angora goats in South Africa. These occurred in sites exposed to sunlight, although he failed to note this point. The areas on which these tumours occur, namely ears, muzzle and perineal region, have either a sparse hair covering or are bare.

Epitheliomata of the ears of cats is commonly seen in animals with white ears brought to the Sydney University Veterinary Clinic (latitude 33° 52′ S.), and this is a common observation of veterinary clinicians in New South Wales (Keep, 1960, personal communication). On the other hand Cotchin (1957) in a survey of feline neoplasms seen at a London clinic (latitude 51° N.) noted the infrequent occurrence of malignant epithelial neoplasms in cats. In his series, six tumours were seen on the ears and six on the body, out of 324 malignant tumours.

So-called " cancer " of the ears of sheep is well known in Australia. It is difficult to obtain accurate figures of its incidence. When cases occur they are either dealt with by cutting off the affected ear, or if advanced, affected sheep are slaughtered for dog food. Animals with well developed tumours are seldom sent to public abattoirs. The opinion is commonly held by graziers that the condition is more common in plains country, much of which is treeless and devoid of shelter from the sun.

This paper records the high incidence of malignant epitheliomata of the ears, muzzle and perineum in a flock of Marino sheep exposed to intense solar radiation in the western district of New South Wales. The occurrence of " cancers " of the skin, and especially of the ears, is common in sheep in these regions and is a minor, but constant cause of wastage in a flock. An increased incidence occurred in one year, 1958, and led to considerable economic loss. Detailed observations were made on cases which occurred during this year.

There are few records of sheep developing neoplasms on areas exposed to ultraviolet irradiation. Beatti (1916) reported a neoplastic condition of the ears of sheep in Argentina, but attributed it to thorns penetrating the ears. Dodd (1923) examined 47 tumours of the ears in Australian sheep of which 32 were epitheliomatous. He observed that such neoplasms were often preceded by a prolonged period of chronic irritation and was inclined to think that infection of the punched out wounds of ear " marks " may play a part. Jackson (1936) reported one tumour on the ear and noted that others occur in the orbital and fronto-parietal areas. Davis and Shorten (1952) reported two cases, both squamous cell carcinomata, one on the eyeball and one on the lower eyelid. Carter (1958) in a short note recorded a high incidence of tumours of the ear in a Merino flock.

Malignant epitheliomata arising in areas covered by wool and thus not exposed to sunlight have been recorded by Feldman (1931), (2 cases, both in the region of the shoulder), and detailed studies have been carried out at this laboratory on an epitheliomatous condition of the wool-covered areas of the body which will be reported in a separate communication.

HISTORY OF THE AFFECTED FLOCK

The property of 16,000 acres on which the outbreak occurred is situated at Burren Junction, latitude 30° S., in the State of New South Wales. This area has a yearly average rainfall of 17·69 inches almost all of which has its origin in thunderstorms which occur principally in the months of November, December, January and February. It has no regular rainfall. The total monthly rainfall, or even more, may fall in a day. Hours of sunlight approach the maximum possible, cloudy conditions leading to rainfall being brief. Average annual hours of sunlight are 3250, the highest average figures occurring in the months of December and January when an average of 310 and 312 hours respectively are experienced.

The rise in incidence of epitheliomata in the flock was first noted in the period January to March 1958. The weather conditions preceding this period were somewhat unusual in that the rainfall for 1957 was 10·57 inches. Drought conditions prevailed. This period came to an end when 6·97 inches of rain, or a third of the average annual precipitation, fell in January 1958. As to be expected, this led to germination of seed and rapid growth of pasture. Following this an outbreak of photosensitization occurred.

The principle pasture species were Coolah grass (*P unicum* sp.), Mitchell grass (*Astrebla* sp.), Flinders grass (*Iseilema* sp.). After summer rains other plants such as Barley grass (*Hordeum leporinum* Link), Nardoo (*Marsilea drummondii*), Camel melon (*Citrullus lanatus*), tar vine (*Boerhavia diffusa*) grow and are available to stock for a short time.

Sheep

The flock consisted of 7840 Merino sheep of mixed ages and except for 828 animals, had been on the property since birth. The ages of animals which had spent all their lives on the station, together with the cancer incidence, is given in Table I.

TABLE I.—*Age Incidence of Tumours in Affected Sheep*

Age group	Number of sheep in group	Number affected	Percentage
1 year old	2350	0	0
2 years old	838	0	0
3 ,, ,,	1139	0	0
4 ,, ,,	976	9	0·92
5 ,, ,,	729	28	3·70
6 ,, ,,	980	32	3·26

(*Note.*—The actual incidence of tumours would be higher than this as the manager of the property had amputated a number of ears and the sheep had recovered. Figures for these were not available.)

The 828 sheep mentioned above were of mixed ages and had been on the property about 12 months. Before that they had been running on another station at Brewarrina to the north of Burren Junction. No tumours were seen in this group even though it did contain some sheep whose ages were comparable with those with highest cancer incidence, that is, five and six years old. The proportion of the various ages in this group could not be determined.

It is important to note that Merino sheep have usually no noticeable natural pigmentation of the ears or the muzzle. However, presumably under the stimulus of sunlight, induced pigmentation of the ears is commonly noted. It is patchy in distribution and varies from light brown to jet black in colour.

Examination of cases

The property was visited in August 1958. All affected sheep had been drafted off and these were examined.

The overall incidence of sheep with tumours was 1·75 per cent, contrasting with the usual incidence of approximately 0·2 per cent in other years.

The ages of affected sheep are given in Table I.

Sites of lesions

In the group of animals examined on the property there was a total of 69 tumours which showed the following distribution :—

Ear . . .	61
Perineal region .	2
Muzzle . .	5
Eye . . .	1

The special features of these sites will be discussed separately.

Ear.—In all instances the site of the tumour was on the outer aspect of the auricle and on the distal three-quarters. Occasionally some had their origin on the anterior edge. There was no association between the site of the tumour and the ear mark which is contrary to the suggestion of Dodd (1923). No tumours had originated on the inner aspect except in two animals in which an ear had been amputated leaving about a third of its length. In these cases tumours were arising from the inner and the outer aspects. There were several instances where tumours had infiltrated around the anterior edge and had affected the inner aspect.

Sites of lesions are consistent with the belief that sunlight is a factor in the aetiology. The outer aspect and the anterior edge of the auricle suffer a much greater exposure to solar radiation than the inner aspect and the posterior edge. Similarly with the more distal part of the ear compared with the proximal portion. In Merino sheep this latter is often wool-bearing and it is also protected by the adjacent neck wool.

However it should be noted that there are differences in the histology of the outer and inner surfaces of the ear. The inner surface has practically no hairs whereas the outer has a hair covering though it may be sparse. The epidermis of the inner surface is rich in sebaceous glands which are often very large (Trautman and Fiebiger, 1957).

Perineal region.—Under normal circumstances this region of sheep is not exposed to sunlight. However, in Australia, it is a common practice to remove pieces of skin from both legs in the area close to the vulva and also across the tail. This results in an area of bare skin larger than normal when it is healed. This is known as the Mules' operation and is done to reduce the incidence of blow fly strike, but exposes the perineal region to the sun, to some degree (Joint Blowfly Committee of the Council for Scientific and Industrial Research, 1943).

Muzzle.—The low incidence of tumours of this site may be unexpected, but sheep in hot sunny weather commonly move about with their heads almost on the ground. Hence exposure is not as great as might at first appear.

Eye.—Remarks regarding the muzzle are relevant to the eye. Furthermore, the top-knot of wool on the fronto-parietal region, and in many cases the wool covering the cheeks, affords considerable protection from direct sunlight.

MORBID ANATOMY

Twenty-eight sheep were submitted for post mortem examination. In all cases the same procedure was adopted. The wool, usually matted with exudate, was clipped off and the lesion was photographed. The animal was then killed. Specimens were taken from the lesion and the regional lymph nodes if these showed any abnormalities. In addition, ears showing keratinized patches of horny growths were collected. A careful examination was made of the lungs and the liver and any lesions suggestive of metastases were retained for histological examination. All material was fixed in 12·5 per cent formalin.

The distribution of tumours in the 28 sheep is given in Table II.

Description of lesions

Ears : The lesions were of two types :—

(*a*) Those in which a long projection of horn-like keratin was the predominant feature (Fig. 1).

TABLE II.—*Sites of Tumours*

Tumour on one ear	16
Tumour on both ears	5
Tumour on one ear and other ear removed	3
Both ears removed	1
Both ears removed and tumour in vulval region	1
Tumour involving the muzzle	2
Tumour involving eyelid	1
Total tumours	40

Note.—In the above table the removed ears, of which there are seven, have been included as if they were squamous cell epitheliomata. This is a justifiable assumption since the manager stated that ears were only removed because of cancer-like lesions.

(*b*) Those in which no such horn was present. These were markedly infected masses of soft, very vascular tissue (Fig. 2).

This subdivision is descriptive only, as type (*b*) is derived from type (*a*) when trauma or infection causes the horny projection to separate from the soft tissues at the base. However, the horny portion obviously shows a greater degree of differentiation, whereas the base is usually undifferentiated so that it is incapable of giving rise to the keratin which is responsible for the form of the projecting lesion. Thus a zone of structurally softer keratin is formed in the progression of the tumour, leading to a natural line of weakness where separation of the horny mass takes place.

Type (*a*) lesion took the form of a large, horny growth. The base was circular, occasionally somewhat pedunculated, a deep red in colour and about 2 cm. in length. It showed an abrupt transition to a dense, hard, projection up to 10 cm. in length (Fig. 1).

Other horny projections were seen on these and other ears in the form of hard, often curled ribbons of keratin which grew up to 4–5 cm. in length. They differed from the above in not having a vascular base. On section they appeared to be keratin built up from a localised hyperplasia.

By the time the horny portion had been shed, as described above, the base had become an actively proliferating, invading epithelioma which progressively involved more and more of the ear. If it had not already been infected it became so at this stage.

EXPLANATION OF PLATES

FIG. 1.—Tumour of ear: type (*a*); vascular, malignant base with peripheral horny projection.
FIG. 2.—Tumour of ear: type (*b*); ulcerated and infected.
FIG. 3.—Tumour of muzzle.
FIG. 4.—Tumour of outer surface of ear showing marked irregular accumulation of keratin and malignant infiltration into subcutis. H. and E. ×30.
FIG. 5.—Ulcerated tumour of outer surface of ear showing malignant infiltration into subcutis, surface ulceration and coagulated exudate. H. and E. ×30.
FIG. 6.—High-power view of area outlined in Fig. 5. H. and E. ×270.
FIG. 7.—Low-power view of section through ear showing hyperkeratosis on outer surface above, and normal inner surface below. H. and E. ×11.
FIG. 8.—High power view of area (A) in Fig. 7 showing hyperkeratosis, irregular, ragged margin of basal epithelial cells and mitotic figures. H. and E. ×270.
FIG. 9.—High-power view of area (B) of normal inner surface. H. and E. ×270.

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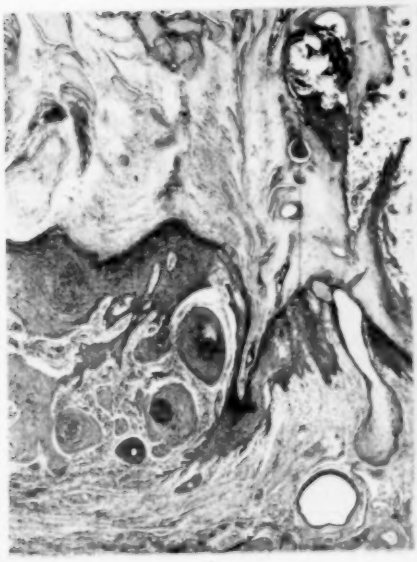
2

Lloyd.

3



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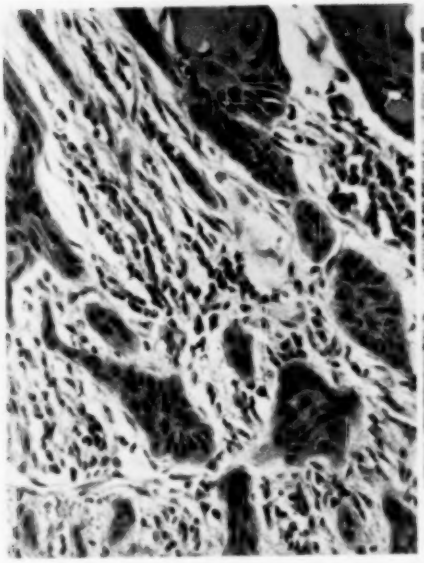

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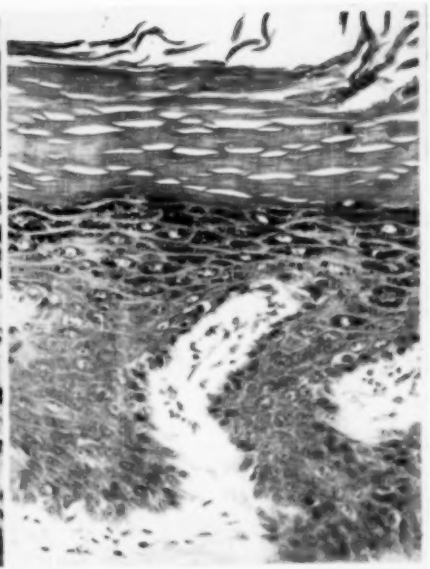

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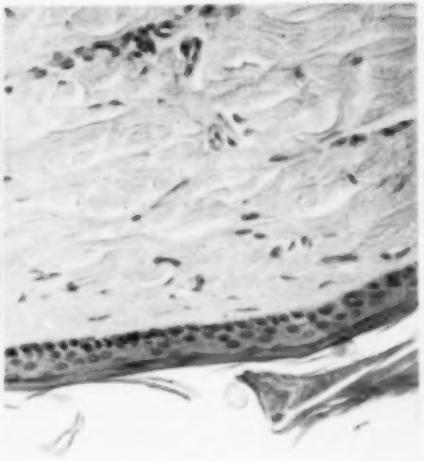

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This was type (*b*) lesion and tended to grow in circumscribed nodules with deep infected crevasses dissecting it. Inflammatory exudate rapidly led to the formation of a hard, black scab which, when lifted, revealed a soft pink highly vascular tissue flecked with pus. Pressure on any part of the lesion caused small amounts of inflammatory exudate to exude through cracks in the scab and from the depths of the crevasses. This exudate stained, moistened and matted the adjacent wool which frequently became fly-blown.

Because of the small number of lesions observed on the muzzle, the eyelids and the vulva, it is not possible to describe their development. These lesions showed the following features :—

Muzzle lesions : One of these was a scab-covered, soft, fissured lesion centred on the area between the two nostrils. It was about 3 cm. in diameter and 2 cm. high. The other tumour, apparently an older lesion, had led to the destruction of the skin and the anterior portion of the turbinate bones which were exposed (Fig. 3). The neoplastic tissue was restricted to the margins of the lesion where it was about 1·5 cm. thick all round.

Eye lesion : The only tumour encountered in this region involved the third eyelid. It was 3 × 2·5 × 1 cm. and was infected. The eye exhibited a marked conjunctivitis.

Vulva tumour : This was situated to the left of and dorsal to the vulva leading to displacement of the anus and vulva to the right. It measured 7 × 6 cm. and had penetrated some 6 cm. into the deeper tissues. It was markedly infected and deeply fissured.

HISTOPATHOLOGY

In selecting tissues for section an attempt was made to include the major mass of the tumour and the junction of the tumour with normal tissue. In most of the ear specimens it was found convenient to remove a rectangular piece 0·5 cm. wide and extending across about half the width of the ear at the mid-ear level. Specimens from the lesions of the muzzle and the perineum were selected so that the margin of the lesion and normal skin would be included in the section. Specimens were embedded in paraffin and cut at 7 μ. All sections were stained with haematoxylin and eosin. Other stains were used where necessary as indicated in the text.

Ear lesions : in all instances the tumours present were squamous cell epitheliomata which varied widely in their degree of differentiation, from tumours showing a high degree of keratinization (Fig. 4) to others with practically no keratin at all. The degree of infiltration also varied considerably. In all cases malignant cells had penetrated into the dermis, but in some they had extended beyond this to involve the subcutaneous tissues (Fig. 5 and 6). In two cases neoplastic cells had penetrated the cartilage and become established in the dermis of the inner aspect of the ear. In both these the epidermis of the inner aspect was still intact and was unchanged except that it was hyperplastic over the area of infiltration. The degree of infiltration was not correlated with the degree of cell differentiation.

The stroma, dense in all specimens, varied in extent. In some instances it was luxuriant and frequently divided the tumour into lobules. In other specimens the tumours were composed almost entirely of epithelial cells.

The epidermis of the outer aspect, that is on the tumour bearing aspect of the auricle, in all cases showed hyperplasia and parakeratosis was frequent (Fig. 7 and 8). In most there was complete loss of hair and in some hyperplastic areas peculiar branching bands of cells somewhat like the fibro-epithelial tumour described by Pinkus (1953) were observed.

A striking feature of the ear lesions was the unchanged state of the epidermis of the inner aspect (Fig. 7 and 9). No tumours were noted on this aspect except where infiltration had occurred from lesions situated on the anterior edge and in the two cases mentioned above where the ear had been amputated. Hyperplasia was the only change observed and this was present in only four of the ears examined. In all other ears the normal 3–4 cell thickness of epidermis was maintained (Fig. 9). This is consistent with solar radiation being an aetiological factor in the pathogenesis of these tumours as the inner apsect of the ear is not exposed to direct sunlight.

All the ulcerated tumours showed considerable inflammation as the result of superimposed infection (Fig. 5). In some tumours in which the keratin cap was still intact, the inflammatory reaction was less marked, while others were free of infection (Fig. 4).

Muzzle, vulva and eye lesions : These were all squamous cell epitheliomata and all were markedly infected.

Connective tissue degeneration.—A number of sections showed an alteration of the dermal connective tissue analogous to the collagen degeneration observed in the skin of man in those individuals who are prone to develop cancers in which sunlight is presumed to be the cause (Unna, 1896 ; Gillman *et al.*, 1955 ; Mackie and McGovern, 1958). In sheep material stained with haematoxylin and eosin loss of structure of the collagen and associated basophilia was noted. These areas stained light blue with Azure A, pale pink with phosphotungstic acid haematoxylin and yellowish-brown with Van Gieson. Sections stained with Giemsa failed to produce the green reaction commonly seen in human material (McGovern, 1961, personal communication).

Evidence of connective tissue degeneration was seen on the dorsal surface in 11 of the 16 tumour-bearing ears suitable for study, and six of twenty tumour-bearing ears showed the same change on the ventral surface. (The discrepancy in the figures arises from four sections in which the tumour occupied the whole of the dorsal surface.) Ten non-tumour bearing ears were available for comparison One of these showed the change on the dorsal surface, possibly representing an early pre-cancerous stage.

Metastases.—In the 28 sheep on which a post-mortem examination was carried out, three cases of metastases to the regional lymph nodes were noted.

The distribution of lesions was as follows :—

Sheep No.	Site of primary lesion	Site of metastasis
X 6	R. ear	R. parotid lymph node which had ulcerated to the surface.
		R. retropharyngeal lymph node.
		R. prescapular lymph node.
	L. ear (this had been removed, presumed carcinomatous)	L. retropharyngeal lymph node.
		L. prescapular lymph node.
		Lung—L. cardiac lobe.
X 1	L. ear	L. prescapular lymph node.
X 20	Both ears	Lymph node.

If tumour progression (Foulds, 1954) in these animals advanced through benign, malignant and metastasing stages, two factors would have tended to reduce the number of metastases found, namely, early death of the animal from infection or fly strike, and early removal of the lesion by the stockowner. Studies in another group of sheep indicate that occasional tumours of the skin give rise to early metastases. In such cases the effect of the above factors on the incidence of metastases would be less marked.

DISCUSSION

Papillomata on the ears of sheep in these latitudes is a common finding and, furthermore, it is usual to find a small but fairly constant incidence of malignant neoplasms of the ears and less commonly of other areas exposed to sunlight. Thus when the incidence increased from the expected 0·2 to 1·75 per cent, the possibilities of an increase in the exposure to the carcinogenic agent or the operation of a co-carcinogenic agent were considered.

It appears highly likely that the carcinogenic agent is sunlight in the condition described here. The neoplasia observed closely parallels the occurrence of neoplasms in man in that tumours occur on areas of the body exposed to direct rays of the sun. Furthermore, in the organ particularly exposed, the ear, it is only the exposed surface which is affected except in some instances where the tumour has infiltrated around the edge of the ear to involve the inner aspect. In addition, the condition occurs in animals with non-pigmented skin in a latitude with prolonged solar radiation.

Having established that there was an increased incidence and that the carcinogenic agent was most likely sunlight, it was important to determine whether an increase in the amount of sunlight may have accounted for the higher incidence. Examination of available meteorological records does not support this. The hours of sunlight in the area referred to tend towards the maximum possible. As stated previously, cloud cover is very unlikely to alter the sunlight since it is unusual to get cloudy days, particularly in summer. Cloud leading to rainfall is short-lived as most of the rain falls in rapidly developing thunderstorms. Thus it seems reasonable to dismiss variation in the hours of sunlight as a factor in the cause of the rise in incidence.

The possibility of the influence of a co-carcinogen was considered. The topical application of a co-carcinogenic substance appeared to be highly improbable. The only unusual occurrence that might have contributed to the rise in the incidence of neoplasia was the photosensitisation that had been observed in the flock. Acute photosensitisation with dermatitis and subcutaneous oedema of the ears and face following the ingestion of a number of different plants at certain stages of their growth (*Hypericum perforatum*, *Panicum decompositum* etc.) occurs commonly in Australian sheep, and also occurs in the disease known as "facial eczema" (Cunningham, Hopkirk and Filmer, 1942) in New Zealand following the ingestion by sheep and cattle of grasses infected with the fungus *Pithomyces chararum*.

There are some experimental evidence and field observations to support the idea that photosensitisation may play a role in carcinogenesis. Büngeler (1937) observed a rise in the incidence of epithelioma of the skin in mice treated with chemical carcinogen by producing photosensitisation with eosin and haemato-

porphyrin. Drabble (1929) noted an association between photosensitisation and epitheliomata of the skin of cattle, and Hore (1961, personal communication) observed unusually high numbers of epitheliomata of exposed parts of the body in sheep in a region where a marked increase in photosensitisation of the "facial eczema" type had occurred.

Thus photosensitisation appears the most likely factor leading to a rise in the incidence of epitheliomata.

There are several pieces of information which are inconsistent with this hypothesis. The first of these is the absence of tumours in the 828 sheep which had been on the property for twelve months. It is certain that some of these were in the susceptible age group of four years and over although the actual number was not known. These would have been subjected both to the initiating agent in similar dosage to the affected sheep throughout their lives and to the promoting agent, photosensitisation, if indeed it is such an agent. One would have expected some cases to appear in these.

Some doubt could be cast on the hypothesis because of the short time elapsing between the operation of the supposed co-carcinogen and the rise in the incidence of neoplasms. Scrutiny of records indicates that although a rise in incidence was noted by the manager during January, February and March 1958, there were 62 cases observed by the author in August 1958 and a further 40 cases in June 1959. As the August 1958 cases would not have survived until June 1959, it is obvious that the latent period was quite long for a substantial proportion of the tumours.

The influence of age on the incidence is of interest. Examination of Table I reveals a lower age limit of about 4 years. Below this there were no cases and above it the incidence was reasonably constant. That there is an influence is consistent with information from other species.

SUMMARY

A sudden rise in the incidence of squamous cell carcinoma of the ear, muzzle and perineum in a flock of Merino sheep is reported.

Attention is drawn to the occurrence of photosensitisation in the flock preceding the rise in incidence and its possible role as a promoting agent in this species is discussed.

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TUMOURS AND OTHER LESIONS INDUCED IN GOLDEN HAMSTERS BY A POLYOMA VIRUS (MILL HILL STRAIN): INDUCTION TIME AND DOSE RESPONSE

F. C. CHESTERMAN AND G. NEGRONI

From the Division of Experimental Biology and Virology, Imperial Cancer Research Fund, Mill Hill, London, N.W.7.

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In 1957 Stewart, Eddy and their colleagues isolated polyoma virus (SEP) in tissue culture from virus induced parotid tumours and leukaemias of mice (Stewart, Eddy, Gochenour, *et al.*, 1957; Stewart, Eddy, Haas and Borgese, 1957; Eddy, Stewart and Touchette, 1958; Eddy, Stewart, Young and Mider, 1958; Stewart, Eddy and Borgese, 1958; Stewart and Eddy, 1959).

In 1959 we isolated a similar polyoma virus (Mill Hill strain) from the spleen of a leukaemic AK mouse (Negroni, Dourmashkin and Chesterman, 1959), and subsequently from the spleen of a C3H mouse with virus-induced leukaemia by the technique of Gross (1951*a* and *b*). The properties of the Mill Hill strain polyoma virus (MHP) are similar to those of the SEP polyoma virus.

When the MHP virus was inoculated into hamsters 1–5 days old it produced vascular lesions, sarcomas or both in the kidney, liver, heart, lung and other tissues. The tumours were already extensive 2–3 weeks after inoculation of the virus. It was therefore important to determine the first appearance of tumour cells and their distribution in different organs. This will be described in the present paper together with the pathological changes obtained by varying the dose of infectious virus inoculated into the hamsters.

MATERIAL AND METHODS

Hamsters.—Golden hamsters have been bred in a closed colony at Mill Hill for the last 8 years, but not by brother-sister mating. Those used were kept in galvanised wire cages and fed on a pellet diet (41*b*) mixed with puppy dog biscuits (Saval) and given water and greenstuff *ad libitum*. Infected litters were examined once or twice daily for clinical assessment, and to remove the dying or dead before they were eaten.

Virus.—The virus preparations were derived: (1) from the spleen of a male AK mouse with spontaneous lymphocytic leukaemia: (2) from the spleen of a C3H mouse with virus-induced leukaemia by the technique of Gross (1951*a* and *b*): and (3) from polyoma-induced tumours of hamsters.

In all cases the virus was isolated by inoculating mouse embryo monolayer tissue cultures with cell suspensions, and the virus was passed serially in similar cultures of mouse embryo tissues. The medium from these cultures contained 10^6–10^7 tissue-culture infectious doses (T.C.I.D.) per ml., and was used fresh or frozen, as infecting virus suspension for the experiments. This virus suspension was centrifuged, with or without subsequent filtration through bacteria-tight

sintered glass filters, and single doses of 0·05 ml. were inoculated intraperitoneally or subcutaneously into baby hamsters 1–5 days old.

Control animals comprised those inoculated with (1) media or media and cells from tissue cultures of normal mouse embryo cells, and (2) cell suspensions of normal hamster kidney.

Histology.—Slices of the organs were fixed in neutral formalin or Zenker's solution, embedded in paraffin and stained with Ehrlich's haematoxylin and eosin.

Initially almost all organs were sectioned and studied, but detailed microscopical examination was later confined to the kidney, liver, heart and lung, and to macroscopic tumours arising at other sites.

RESULTS

Post mortem examination

MHP virus produces tumours or vascular lesions or both in over 90 per cent of hamsters inoculated parenterally with 10^6–10^7 T.C.I.D. Most of the hamsters died between 12 and 31 days after inoculation.

At autopsy the livers showed many circumscribed blood-red spots up to several millimeters in diameter, flush with the surface or projecting above it. The intermediate liver was normal or pale. On cutting the liver, blood-stained fluid escaped from the cysts, giving it a honeycomb appearance (Fig. 1). Some of the larger cysts contained ante mortem thrombi. In females minute haemorrhagic cysts could sometimes be seen in the uteri and ovaries.

The kidneys were enlarged and pale, and often contained several white nodules projecting above the surface. Less frequently there were small subcapsular haemorrhages. In older lesions, the cut surface showed firm white tissue replacing the kidney, but only partially involving a thin outer rim of the cortex and sparing the papilla which is relatively long in the hamster (Chesterman, 1961). The heart often showed tumour nodules in the auricular and ventricular walls (Fig. 2). These tumours projected into the heart chamber, or into the mediastinum with only a thin pedicle attached to the heart. Similar tumours adhered to the outer coats of the veins near their entrance to the heart. In moribund animals bloody fluid could sometimes be observed around the mouth and nose, and at necropsy blood was present in the trachea and main bronchi. The lungs appeared normal or showed multiple small red spots, sometimes projecting above the pleural surface. In some animals surviving for more than two weeks small grey tumours were also present, either separately or in the centre of some of the red lesions. Death was often associated with haemorrhage from rupture of one or more haemorrhagic liver lesions, extensive sarcomatous involvement of the kidneys, or both.

Histology

There were two main groups of lesions : (1) tumours, (2) vascular lesions.

Tumours.—Morphologically the tumours were spindle-cell sarcomas composed of darkly-stained cells varying in size and showing nuclear irregularity. In the kidney they appeared first in the cortico-medullary junction and later involved the inner cortex and outer medulla destroying the kidney tubules (Fig. 3). Multiple nodules could sometimes be seen in the outer cortex. Morphologically similar sarcomas were present in the heart muscle or valves. As these tumours

increased in size the centre underwent necrosis. Similar sarcomas were sometimes present in the liver, particularly near the hilum where the inferior vena cava passes through the diaphragm, in the lungs, and in one instance attached to the outer bowel wall, as described by Stoker (1960) for the B.P. (Toronto strain) virus.

In three cases only were subcutaneous sarcomas seen. One about 3 mm. in diameter occurred near the external auditory meatus of a hamster dying from internal lesions 20 days after inoculation. Two more were found in animals dying on the 79th and 145th day after inoculation. Subcutaneous sarcomas were more frequent when lower doses of virus were used (*vide infra*) or after a long latent period when adult animals were inoculated with high doses of virus. This latter finding is similar to the results of Defendi (1960).

Areas of connective-tissue mesenchymal hyperplasia were sometimes present in the testis, adrenal cortex, ovary and meninges.

Another type of tumour formed by proliferation of cells lining the blood lakes and resembling an angiosarcoma was present in the liver and sometimes as emboli in the lungs.

Vascular lesions.—These occurred frequently in the liver as dilatation of the sinusoids and ends of the interlobular portal and intralobular central veins, forming blood lakes with disruption and loss of adhesion of liver cells so that the cells formed clumps within the lakes (Fig. 4). Focal areas of necrosis unaccompanied by surrounding inflammatory cell infiltration but sometimes associated with haemorrhage or small intrasinusoidal thrombi were also seen. Occasionally fragments of the larval stage of *Cysticercus fasciolaris* were present.

In the lungs there were areas of intra-alveolar or interstitial haemorrhage, or both, with consolidation and vascular dilatation similar to that seen in the liver but usually not so extensive. The walls of some of the distended vessels appeared to contain dissecting aneurysms.

Emboli in the lung were either " normal " liver cells or clumps of sarcoma or angiosarcoma cells (Fig. 5). In a few animals vascular dilatation and sometimes haemorrhage were present in the brain, ovary, uterus, bowel and subcutaneous tissues.

Induction time

In order to study the development of the tumours and vascular lesions, groups of 2–5 animals were killed at 2–3 day intervals after virus inoculation. Three

EXPLANATION OF PLATES

FIG. 1.—Liver of female hamster aged 22 days inoculated with MHP virus when 2 days old. Honeycomb appearance. ×6·5.

FIG. 2.—Heart of male hamster aged 27 days inoculated with MHP virus when 2 days old. Multiple nodules replace the ventricular walls. ×6.

FIG. 3.—Kidney of male hamster aged 7 days, 6 days after inoculation with MHP virus. Early tumour growth. ×315.

FIG. 4.—Liver of male hamster inoculated with MHP virus when 2 days old. Clumps of liver cells lie in a blood lake continuous with a central vein. ×95.

FIG. 5.—Lung from male hamster aged 27 days, inoculated with MHP virus when 2 days old. Top right, a clump of normal liver cells; botton left, a clump of tumour cells. ×650.

FIG. 6.—Liver of male hamster aged 27 days inoculated with MHP virus when 2 days old. Proliferation of mesenchyme cells between parenchymal liver cells and the wall of the sinusoids. ×650.

All sections stained H. and E.

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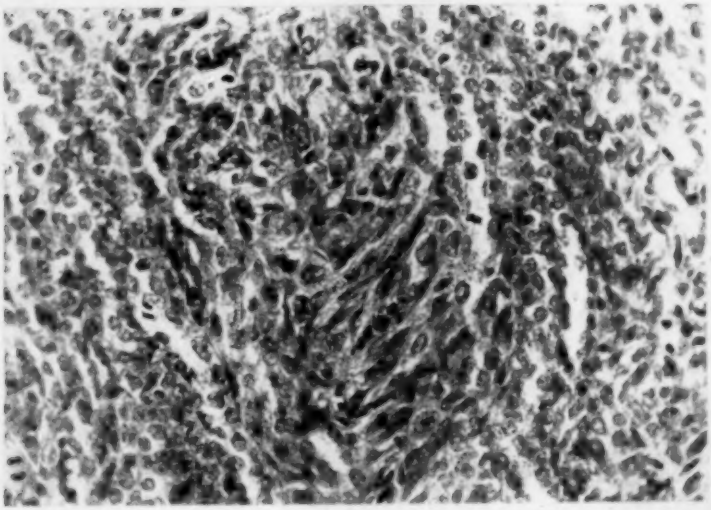

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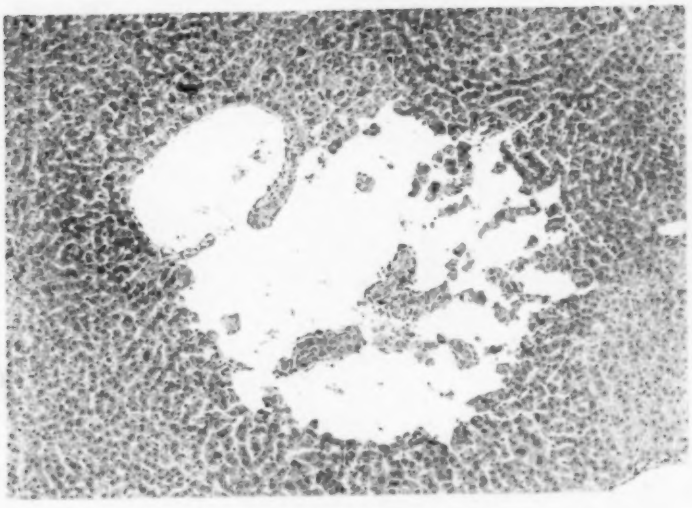

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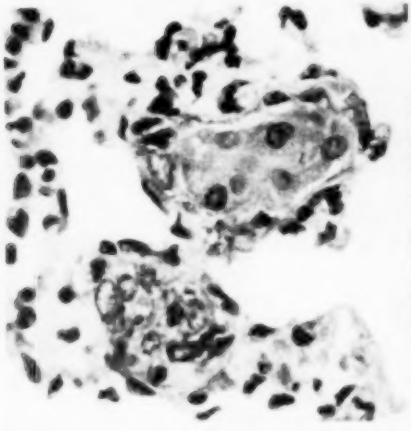

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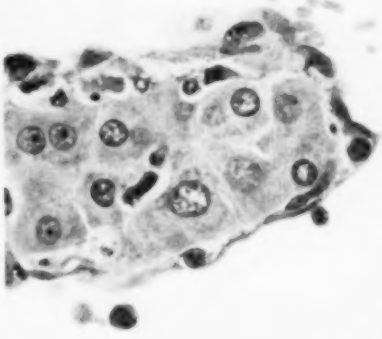

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groups of hamsters were used ; one inoculated at one day old, another at two days old, and the third at five days old. Table I shows the combined results of the first two experiments. Similar lesions were found in hamsters inoculated up to 5 days old.

TABLE I.—*Distribution of Tumours and Vascular Lesions in 66 Hamsters Inoculated When Newly Born with Polyoma Virus (Mill Hill Strain) and Killed at Various Intervals*

Time after inoculation (days)	Number of hamsters positive/ hamsters examined	Number of hamsters with: Tumours of: Kidney	Heart and vessels	Liver	Lung	Subcutaneous tissue	Vascular lesions of liver and/or lung
3	2/12	1	0	0	0	0	1
6	9/10	5	5	0	1	0	7
8–9	11/11	10	3	6	2	0	8
12–15	20/21	19	18	16	13	0	20
16–25	10/10	10	8	6	5	1	10
79	1/1	1	1	0	1	1	1
145	1/1	0	1	0	0	1	0

In one of the 12 animals examined small microscopic foci of tumour cells were present in the kidney three days after inoculation. On the 6th day after infection the regular appearance of the intertubular mesenchyme was replaced by an irregular pattern of plump spindle-shaped darkly-staining tumour cells (Fig. 3). There were no gross degenerative changes in the cells in the kidney in the early lesions, but necrosis of the central parts of the tumours occurred later, sometimes associated with vascular thrombosis.

By the 6th day but sometimes later, focal proliferation of histiocytes occurred in the heart sometimes associated with small areas of degeneration of muscle fibres.

The earliest change observed in the liver was ectasia of the veins and sinusoids to form blood " lakes ", followed by detachment of the liver cells into the lumen of the sinus ; individual or clumped parenchymal cells were surrounded by reticular or primitive mesenchymal cells, or both (Fig. 4 and 6). Later these connective-tissue cells proliferated giving rise to the tumours described, but these were never so extensive as those seen in the kidney or heart. Organised or unorganised thrombi occurred in the blood lakes. Embolic tumours appeared in the lung 6–9 days after infection.

By the 12th day extensive tumours were present in the kidney and heart, and the liver was studded with blood lakes.

Dose response

The above description applies only to hamsters inoculated with a very large dose of virus (10^6–10^7 T.C.I.D.). The pathological changes in animals inoculated with decreasing doses of virus were studied in relation to the induction period. Serial tenfold dilutions of infective tissue-culture media were inoculated into 1-day-old hamsters and also into 5-day-old hamsters and into mouse embryo tissue cultures. In the latter 0·1 ml. virus proved to be infectious to a dilution of 10^{-5}.

Observations and histological examinations were made up to 620 days after infection. In a similar experiment Rowe *et al.* (1959) killed their animals only up to 35 days after inoculation, and their results gave an incomplete picture.

The results of the virus titration in hamsters are shown in Tables 2 and 3. The interval between inoculation and death is prolonged with decreasing virus dose (Table 2).

TABLE II.—*Dose Response to Polyoma Virus (Mill Hill Strain) In Hamsters Inoculated When 5 Days Old*

Dose of virus injected (TCID*)	Positive animals			Negative animals	
	Number of animals inoculated	Number died or killed because of lesions or tumours	Mean time to death (days)	Number without tumours or vascular lesions†	Mean time to death (days)
10^5	3	3	27	0	—
10^3	6	5	109	1	620
10^2	11	9	180	2	264
10^1	12	5	261	7	563

* TCID = Tissue culture infectious dose.
† Killed or dead from other causes.

With 10^5 T.C.I.D. the average time to death was 27 days, rising progressively to 261 days with 10 T.C.I.D. This negative correlation is significant ($r = -0{\cdot}65$, $p < 0{\cdot}01$). One hamster inoculated with 10^3, two with 10^2 and seven with 10 T.C.I.D. were negative at periods between 180 and 620 days after inoculation. The incidence of vascular lesions and tumours of the internal organs declined with decreasing dose of virus but tumours of subcutaneous tissue were found more frequently (Table 3). These tumours were not necessarily at the inoculation site.

TABLE III.—*Dose Response to Polyoma Virus (Mill Hill Strain) and distribution of Vascular Lesions and Tumours in Hamsters Inoculated when 5 Days Old*

Dose virus injected (TCID)	Number of animals with						Number of tumours localised to inoculation site or subcutaneous tissue
	Tumour of				Vascular lesions of		
	Kidney	Heart and vessels	Liver	Lung	Lung	Liver	
10^5	3	3	1	1	2	3	0
10^3	0	2	3	1	4	5	4
10^2	0	0	1	1	1	1	9
10^1	0	0	0	0	0	1	4

DISCUSSION

Time of appearance of lesions

Various combinations of vascular lesions with sarcomas, haemangiosarcomas, or both, can be regularly induced in the kidney, liver, heart and lungs of newborn hamsters by parenteral inoculation of high doses of polyoma virus (Mill Hill strain). Some of these lesions appear as early as the 3rd day after infection and are responsible for the death of the majority of the animals within the first 4

weeks. With smaller doses, animals survive for longer periods, and tend to develop sarcomas of the soft tissues with few vascular lesions or sarcomas of the internal organs.

Tumour induction by virus

The inverse relation between dose of infectious virus and duration of the induction period strongly supports the view that virus multiplication does not occur in the infected hamster. This is in agreement with the data of Sachs and Winocour (1959), Habel and Atanasiu (1959), Negroni and Chesterman (1960), Roizman and Roane (1960) and Vogt and Dulbecco (1960), who found that polyoma tumour cells are not all necessarily virus-producing. This is also in agreement with the finding in our experiments that the number of tumour sites decreases with diminishing doses of virus as in the experiments of Stoker (1960) and with the finding that the action of the virus is a local, rather than a systemic one (Stanton, 1960).

Virus is present in the affected organs, but only in small quantities, as early as the 3rd day after injection (Negroni and Chesterman, 1960). This suggests that the virus is directly responsible for the primary change in the cells that leads to the formation of the tumour, and the very early appearance of the tumours precludes any sequence of initiation and promotion.

Histopathology

The degenerative changes in the nuclei of the interstitial cells in the kidney that occur shortly after inoculation (Ham *et al.*, 1960) were seen only infrequently. Intranuclear inclusions have not been observed in the kidney tumours.

The virus-induced hamster tumours have the same biological characteristics as other "spontaneous" tumours in hamsters in that they are invasive, transplantable within a closed colony and sometimes give rise to metastases. In mice, tumours, in the accepted sense of the word, can be obtained from epithelial as well as connective-tissue cells after infection with polyoma virus.

Histogenesis

Although the precise histogenesis of the hamster tumours has not been fully elucidated they are probably sarcomas or haemangiosarcomas, possibly arising from the primitive mesenchyme in relation to blood vessels (Fig. 6). Some of the lesions are not unlike those seen in Kaposi's sarcoma in man.

Rabson and Kirschstein (1960) have shown that intracranial sarcomas produced by polyoma virus in Syrian hamsters probably arise from the adventitia of small meningeal and cerebral vessels, as well as from the connective tissue in the pia mater.

The kidney of the newborn hamster appears to be a comparatively primitive structure. It is possible that it is in a favourable morphological and biochemical stage of development for tumour formation with polyoma virus. In fowls, for example, carcinomas can be induced in the immature kidney with ES4 and MH2 virus (Carr, 1959).

The mechanism underlying the development of the blood lakes in the liver is unknown. Emboli of normal liver cells have been seen in the lungs of one control animal in the absence of any gross pathological change.

No spontaneous disease similar to that described has yet been observed in hamsters.

It is interesting to note that the liver lesions are similar to those produced in mice by the injection of urethane (Kawamoto *et al.*, 1961) and in young chicks by Rous sarcoma virus (Duran-Reynals, 1940). Haemorrhagic cysts in various organs occur when Rous sarcoma virus is inoculated into embryonic or into newly born rats (Zilber, 1961). Terracini, Porta and Maxwell (1959) describe a transplantable haemangiosarcoma in one of thirty hamsters fed with 4-dimethylaminoazobenzene, but no relationship to the treatment was established. This tumour resembles some of the lesions seen in the hamsters infected with MHP virus.

SUMMARY

Injection of large amounts of polyoma virus (Mill Hill strain) produces in hamsters a variety of sarcomas, vascular lesions, or both. These begin to appear in the kidney as early as the 3rd day after inoculation. Smaller amounts of virus induce tumours mainly in the soft tissues after a long latent period. The histogenesis of the tumours is discussed.

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EFFECT OF IMMUNOLOGICAL ATTENUATION ON CELL DOSAGE REQUIRED TO ESTABLISH SINGLE OR DOUBLE TUMOUR HOMOGRAFTS

H. A. S. VAN DEN BRENK

From the Radiobiological Research Unit, Cancer Institute Board, Melbourne, Australia

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THE conditions which govern the establishment and growth of multiple tumour cell deposits in the affected animal are important in both experimental and clinical situations. In the latter the removal or ablation of a single tumour not infrequently seems to initiate the appearance of new foci or an altered rate of growth of other remaining deposits. This applies particularly to the removal of a primary tumour and the appearance and rate of growth of metastases. A similar experience has been described in experimental tumour-host systems by Ehrlich (1908) and more recently by others (Marie and Clunet, 1910; Tyzzer, 1913; Schatten and Kramer, 1958). In experimental situations however, strict tumour-host immunological compatibility seldom exists, and indeed a homologous relationship has applied to much of the earlier experimental work dealing with this problem (see review by Woglom, 1929). Also tumour inoculations usually have been made with solid fragments, and the quantitative assessment of results is difficult.

In the present work cell titration techniques have been used in a homologous tumour-host system to determine the number of cells required to induce 50 per cent of single tumours (ED_{50}) in mice for both single (one leg) and double (two leg) inoculations, and similarly the ED_{50} for the induction of double tumours resulting from double inoculations. Similar determinations of the various ED_{50} cell doses were made for the same system after immunological depression of host mice with whole body X-irradiation.

MATERIALS AND METHODS

Animals and tumour

Walter and Eliza Hall hybrid male mice weighing 30–40 g. were used as recipient animals for tumour cell inoculations. Ehrlich ascites tumour, hyperdiploid line ELD Lettre, 46 chromosomal mode was used. It had been passaged at weekly intervals as an ascites growth in C3H strain mice for the past 4 years in these laboratories. All experimental animals were housed in an air conditioned room maintained at $20° \pm 1°$ C.

Tumour inoculations and titrations

A 5–7 day growth of ascites fluid was removed, a cell count was made on this fluid using the eosin exclusion technique of Schrek according to Hoskins, Meynell and Sanders (1956) to determine the viable cell index. Samples containing less

than 95 per cent viable cells were not used in titrations. Suitable dilutions of the cells were made in ice cold Tyrode solution to give doses of 10^1–10^4 cells per 0·2 ml. of fluid for inoculation. Each inoculation (0·2 ml.) was made intramuscularly in the thighs of recipient mice, just proximal to the knee joint.

Groups of mice received either a single inoculum (right or left leg) or a double inoculum (both legs), as follows :—

Group A (unirradiated) .	one leg (right or left) inoculated ;
Group B (irradiated) .	one leg (right or left) inoculated ;
Group C (unirradiated) .	both legs inoculated ;
Group D (irradiated) .	both legs inoculated.

In each group the mice were subdivided into six subgroups, each subgroup consisting of 8–10 mice and six cell doses in the range 10^1–10^4 cells were used for inoculation of the subgroup mice. In groups C and D both legs of individual mice were inoculated with the same number of cells, but the doses varied amongst subgroups as for single inoculations.

Groups B and D mice received a mean dose of 450 rads whole body X-irradiation, 24 hours preceding inoculation, using an X-ray source operated at constant potential, the factors being 250 kV, 15 mA, 30 cm. FSD, 1 mm. Cu HVL and dose rate in air of 300 r per minute. Animals were irradiated with maximum back scatter provided by bolus packing and the doses calculated accordingly.

Analysis

The number of mice in each subgroup developing palpable tumour within six weeks was scored. Mice dying during this period were excluded from the final analysis. In this experiment no animal developed a tumour which spontaneously regressed. An analysis was made (i) of the fractions of animals in each subgroup of Groups A and B (single leg inoculations) which developed a tumour, (ii) the fraction of mice in each subgroup of C and D (double leg inoculations) which developed at least one tumour and (iii) the fractions in subgroups of C and D which developed two tumours (i.e. both limbs). Using the approximation method of Finney (1952), probits were calculated and a regression analysis made of the tumour incidence for the various groups. The mean inoculum cell dose (ED_{50}) which resulted in a 50 per cent incidence of tumours was calculated for each of the 4 groups.

RESULTS

The relevant regression equations, statistics and analysis of results for the various groups are shown in Table I.

The findings indicate that for immunologically competent mice subjected to the tumour homograft inoculation, the number of cells which had to be grafted in each inoculum site to give a 50 per cent incidence of tumour bearing animals (ED_{50}) was not significantly different if one or two inoculum sites (legs) were used, namely, 282 and 355 cells respectively, although the animals inoculated in both legs could be considered to have had an increased chance of developing one tumour, since they received a total inoculum dose of double the number of cells. However, each cell inoculum has to be increased approximately tenfold (ED_{50} = 3162 cells) for 50 per cent of animals inoculated in two legs to develop two tumours.

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TABLE I.—*Tumour Incidence Following Single and Double Inoculations of Hybrid Mice made with Ehrlich Ascites Tumour, with or without Previous Whole body X-irradiation (450 rads). Analysis Based on the Probit Regression* $Y = m (\pm Sm) x + a$

Group	Regression $Y = m (\pm Sm)x + a$	$\chi^2(n)$† (p)	ED_{50} ($\pm$SE)	t (p)
A (Unirradiated—single leg inoculation)	$Y = 1{\cdot}9 (\pm 0{\cdot}11)x - 0{\cdot}34$	2·26 (6) $p = 0{\cdot}65$	282 ± 71*	—
B (Irradiated—single leg inoculation)	—	—	< 10	—
C (Unirradiated—double leg inoculation)	(i) $Y = 1{\cdot}3 (\pm 0{\cdot}16)x + 1{\cdot}56$ (one or more tumour per animal)	1·75 (4) $p = 0{\cdot}45$	355 ± 130*	—
	(ii) $Y = 2{\cdot}0 (\pm 0{\cdot}11)x - 2{\cdot}00$ (two tumours per animal)	4·96 (6) $p = 0{\cdot}30$	3162 ± 800	3·3 ($p < 0{\cdot}05$)
D (Irradiated—double leg inoculation)	(i) $Y = 3{\cdot}1 (\pm 0{\cdot}09)x + 0{\cdot}62$ (one or more tumour per animal)	0·32 (3) $p > 0{\cdot}8$	24 ± 5	—
	(ii) $Y = 3{\cdot}9 (\pm 0{\cdot}08)x - 1{\cdot}32$ (two tumours per animal)	0·50 (3) $p > 0{\cdot}7$	42 ± 9	1·2 (n.s.)

* Difference not significant ($p = 0{\cdot}6$).

† The statistic $\left(\chi^2(n) = \frac{(r - nP)^2}{nP(1 - P)}\right)$ is used to determine heterogeneity of departure from the fitted probit line.

Since the recipient mice were not preimmunised with the Ehrlich tumour antigens, and since considerable cell multiplication and antigenic stimulation would take place before significant antibody production could be expected, it was considered that these difference in the ED_{50} values could be attributed to quantitative differences in development of the antigenic stimulus. To test this hypothesis, a similar series of inoculations was made in recipient mice previously given whole body X-irradiation (450 rads) to depress the immune response. It is seen that the various ED_{50}s were greatly reduced, and that the difference between single and double tumours developing in mice which received two inoculations, was no longer significant.

In Table II are shown the times of appearance of palpable tumours following the inoculation of either 5×10^2 or 10^3 EAT cells in groups unirradiated and irradiated recipients. It is seen that both single and double tumours appeared earlier in the irradiated groups. In the mice which ultimately developed double tumours, each of the two tumours appeared at much the same time and this finding applied to both unirradiated and irradiated groups.

DISCUSSION

The results show that for tumour homografts the development or progression of a single focus of tumour is clearly dependent on other cells of the same tumour

TABLE II.—*Incidence of Palpable Tumours Scored Weekly in Surviving Unirradiated and Irradiated Mice after receiving either Single or Double Inoculations of EAT cells. The Fraction of Mice with Palpable Tumours is Designated T_0 (no tumours), T_1 (One Tumour only) and T_2 (Two Tumours) respectively*

	Number EAT cells inoculated	Time after inoculation			
		7 days	14 days	21 days	28 days
Unirradiated group	10^2 cells in one leg	T_0 (10/10)	T_0 (7/10) T_1 (3/10)	T_0 (2/10) T_1 (8/10)	T_0 (3/10) T_1 (7/10)
	10^2 cells in each leg	T_0 (10/10)	T_0 (8/10) T_1 (1/10) T_2 (1/10)	T_0 (1/9) T_1 (5/9) T_2 (3/9)	T_0 (0/9) T_1 (6/9) T_2 (3/9)
	5×10^2 cells in one leg	T_0 (10/10)	T_0 (10/10)	T_0 (7/10) T_1 (3/10)	T_0 (3/10) T_1 (7/10)
	5×10^2 cells in each leg	T_0 (10/10)	T_0 (10/10)	T_0 (5/10) T_1 (4/10) T^2 (1/10)	T_0 (5/9) T_1 (3/9) T_2 (1/9)
Irradiated (500r) group	10^2 cells in one leg	T_0 (9/9)	T_0 (0/8) T_1 (8/8)	T_1 (8/8)	T_1 (8/8)
	10^2 cells in each leg	T_0 (9/9)	T_0 (0/9) T_1 (0/9) T_2 (9/9)	T_2 (8/8)	T_2 (7/7)
	5×10^2 cells in one leg	T_0 (8/8)	T_0 (0/6) T_1 (6/6)	T_1 (6/6)	T_1 (6/6)
	5×10^2 cells in each leg	T_0 (10/10)	T_0 (0/10) T_1 (0/10) T_2 (10/10)	T_2 (10/10)	T_2 (9/9)

being present and developing in the host animal at the same time. Furthermore it would seem that this is a mechanism which is largely based on an immune response, since the phenomenon disappears following depression (attenuation) of the immunological response of the host animal by whole body irradiation. The implications of this finding are of considerable importance to experimental techniques used in oncology which depend on the establishment of multiple cell foci following intravenous inoculation of cells as a method of simulating metastatic spread. Whether a similar reservation applies to strictly isologous systems such as that of Hewitt and Wilson (1959) is doubtful. However, a review of previously reported results of cell titration in certain isologous systems, has shown that ED_{50} doses as high as 10^7 cells were reported (van den Brenk, 1961). The criticism arises that immunological incompatibility may largely account for the magnitude of such values. That whole body irradiation is a useful experimental expedient in reducing the homograft reaction has been shown by recent studies (Mazurek and Duplan, 1959; Cohen and Cohen, 1960; van den Brenk, 1961).

In helping to explain certain growth phenomena seen in human tumours, the results of experimental animal studies need careful assessing in terms of homograft reactions (Woglom, 1929). In the early work of Marie and Clunet (1910) and Tyzzer (1913) who reported that partial excision of implanted tumours in mice was followed by enhanced growth of metastases, homografts were used. In the important studies of Schatten (1958) solid tumour transplants of melanoma S–91 and DBA sarcoma 49 were made in either DBA or C × DBA hybrid mice. Schatten showed that removal of such primary tumours resulted in the establishment and rapid growth of large numbers of latent pulmonary metastases. This phenomenon was not dependent on the surgical trauma (Schatten and Kramer, 1958) and was considered to show that "a primary tumour of sufficient size in-

hibits the development and growth of its distant metastases". Furthermore Schatten considered that the "majority of the metastases in these tumour-host systems would have been dormant or would have succumbed if the primary tumour had not been removed". It is unfortunate that immune reactions may have influenced these results. Similarly in experimental studies which claim to have shown that irradiation of a primary tumour causes an increase in the development of metastases (Kaplan and Murply, 1949; von Essen and Kaplan, 1952; Kaae, 1953), proper evaluation was not made of the effect of the irradiation on immunological compatibility, since the most careful local irradiations of tumours in small animals, are accompanied by a substantial whole body dosage contribution (van den Brenk, 1961). In the work of Olch, Eck and Smith (1959) the incidence of pulmonary metastases following local irradiation of melanoma S–91 in hybrid C × DBA mice with 3000 r was not consistently increased nor decreased. For a hamster lymphosarcoma Greene (1959) reported that surgical removal of the primary tumour was followed by an increased incidence of widespread metastases. Biopsy or removal of normal tissue, *per se*, had no effect on metastases. On the other hand Greene reported that for two transplants made in different sites, whilst the one did not influence the growth of the other, two such growing tumours actually reduced the incidence of metastases from 10 per cent to nil, and that removal of one of the two primary tumours raised the metastatic incidence to the basal 10 per cent level for one tumour! The results of experiments of Flintjer and Mefferd (1960) using a transplantable fibrosarcoma in Sherman strain rats, are difficult to assess, but in general demonstrate that viable tumour tissue, whether it was implanted or not, caused resistance to further grafting in test animals.

In conclusion, the factors which determine the balance of growth rates for multiple tumours in the same individual, seem to be of considerable importance to both clinical and experimental studies. However the experimental approach to the problem should particularly aim to control two critical factors, namely the inoculation cell dosage and the tumour-host immunological relationship. Cell titration techniques are considered desirable as distinct from solid tumour transplants. Also, in isologous systems the determination of ED_{50} doses is essential and immunological attenuation by whole body irradiation is an added safeguard in evaluating the participation of immune reactions in the observations made. In this way, humoral factors which are distinct from antigen-antibody reactions and which influence the development and growth rate of tumours in the body, may be demonstrated and their nature established. The results reported in this paper suggest that for the tumour homograft studied, such balance of growth is largely dependent on an immune reaction, and for this reason does not clarify the factors which determine balance of growth in spontaneous human tumours. Similarly the almost complete absence of data relating to ED_{50} values for the cells of spontaneous human tumours, greatly hampers the evaluation of similar factors in humans in respect to both the natural history of the disease and its treatment by radiotherapeutic and other means.

SUMMARY

Titration of hyperdiploid Ehrlich ascites tumour cells in mice as single leg inoculations showed that 282 cells were required to induce 50 per cent of tumours (ED_{50}). When double leg inoculations were made, the ED_{50} for single tumour development was 355 cells, and not significantly increased. However the cell

dose required for inoculation of each leg for 50 per cent of the animals to develop two tumours was increased tenfold to 3162 cells. Whole body X-irradiation of mice preceding inoculation, to reduce the immune response greatly reduced the ED_{50} value to <10 cells for single inoculations, whilst the ED_{50} doses for single and double tumour establishment after double inoculations were 24 and 42 cells respectively. These findings are discussed in relation to clinical and experimental situations where the establishment and growth of multiple neoplastic foci in the single host are to be evaluated.

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EXPERIMENTS ON THE CARCINOGENICITY AND REACTIVITY OF β-PROPIOLACTONE

C. E. SEARLE

From the Cancer Research Laboratories, Department of Pathology, The Medical School, Birmingham 15

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β-PROPIOLACTONE (3-hydroxypropionic acid lactone, $\underline{O.CH_2.CH_2}C : O$) is a highly reactive compound of simple structure which is now available in quantity. It has many possible uses as an organic synthetic agent, and has been recommended for sterilising plasma (Hartmann, LoGrippo and Kelly, 1954) and arterial grafts (Rains *et al.*, 1956) and as a toxoiding agent in place of formalin (Orlans and Jones, 1958). It is therefore of importance that β-propiolactone, besides being a vesicant, is also a mutagenic agent (Smith and Srb, 1951), has been found to cause sarcomas after injection in arachis oil into rats (Walpole *et al.*, 1954) and gives rise to papillomas and squamous cell carcinomas when applied in acetone solution to mouse skin (Roe and Glendenning, 1956). β-Propiolactone is thus suspect as a carcinogen to man, and Roe and Salaman (1958) have questioned the advisability of using it for arterial graft sterilisation.

Apart from such practical considerations, it is of considerable theoretical interest that relatively simple compounds such as β-propiolactone and various ethyleneimines should have carcinogenic activity. It is possible that studying the biological reactivity of these substances, which Walpole *et al.* (1954) suggested may act as carcinogens *per se*, could give more useful information about the carcinogenic process than has hitherto been obtained from work on the aromatic hydrocarbon carcinogens.

Dickens and Jones (1961) have now shown that a range of other lactones can induce sarcomas when repeatedly injected into rats. Structural features of the active compounds are either a highly strained four-membered ring as in β-propiolactone and penicillin G, or a five-membered ring with double bonds in the 2- and/or 4-positions, as in patulin and penicillic acid. A number of these compounds have also antibiotic and growth inhibitory activity, and are inactivated by cysteine.

Among other reactions of β-propiolactone those with biological sulphydryl groups are of particular interest. Searle (1961) investigated the effects of β-propiolactone on the sulphydryl and disulphide groups of egg and serum albumins by polarography of the denatured protein solutions at intervals after adding the lactone. It was found that β-propiolactone caused immediate destruction of polarographically active groups in the albumins, but that these effects were reversible when the solutions were warmed or allowed to stand at room temperature.

This could indicate that the main product from the reaction of β-propiolactone with cysteine residues in the protein has a hydrolysable thioester grouping. In

a preliminary report Dickens, Jones and Williamson (1956) also named as a thioester the product $C_6H_{11}O_4NS$ which they obtained from the reaction between cysteine and β-propiolactone in neutral solution, but it has now been identified (Dickens and Jones, 1961) as the thioether S-2-carboxyethylcysteine, HOOC.CH_2.CH_2.S.CH_2.CH(NH_2)COOH.

The formation of thioester and thioether involve opening of the β-lactone ring by fission of different C-O bonds as illustrated in Fig. 1. β-Propiolactone is known to undergo both types of reaction with OH-groups, methanol for instance yielding methyl 3-hydroxypropionate in the presence of alkali and 3-methoxypropionic acid in its absence (Fieser and Fieser, 1956). One would, perhaps, expect an alkylation process with thioether formation to have more relevance to carcinogenesis than the formation of a thioester which could readily hydrolyse.

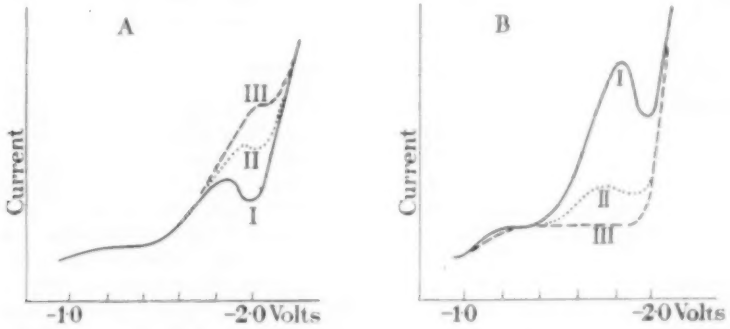

FIG. 1.—Reaction of β-propiolactone (I) with cysteine derivative (II) by fission at (a) to yield a thioether (III) or at (b) to yield a thioester (IV). The latter would be expected to hydrolyse to give (II) again and 3-hydroxypropionic acid (V). Reaction (a) is known to occur with free cysteine. Reaction (b) it is suggested, may occur when cysteine is in protein combination.

In some preliminary small-scale experiments the reaction between β-propiolactone and cysteine was followed by paper chromatography in two solvents (aqueous phenol—NH_3 and aqueous butanol—pyridine, 1 : 1 : 1). The reaction mixtures contained a product believed to be S-2-carboxyethylcysteine since it had the same R_f value as an authentic specimen (L. Light & Co.) in each solvent, both with and without prior oxidation of reaction mixture and marker by hydrogen peroxide. This result is as expected in view of Dickens and Jones' (1961) isolation of S-2-carboxyethylcysteine from the reaction at pH 7.

In one experiment, however, it was noticed that the nitroprusside reaction for –SH, which had become negative on adding the lactone, became positive again when the solution was allowed to stand. This suggested that under some conditions cysteine could also yield the thioester on treatment with β-propiolactone. To investigate this possibility the reaction between the compounds has been carried out in controlled neutral, mildly alkaline and mildly acid conditions, the course of the reaction being followed polarographically as in the albumin experiments already reported (Searle, 1961). In view of the destruction of protein-bound cystine, as well as cysteine, which is thought to have occurred in

albumin experiments, the action of β-propiolactone on free cystine has been similarly investigated.

If the carcinogenicity of β-propiolactone is in fact associated with alkylation by the 2-carboxyethyl group, it seemed possible that β-(or 3-)bromopropionic acid, which reacts with cysteine at pH 6–7 to give S-2-carboxyethylcysteine (Schöberl and Wagner, 1956), might also show some carcinogenic activity. It has accordingly been tested on mouse skin in experiments reported below. Tests thought desirable to confirm the carcinogenicity of β-propiolactone on a larger number of mice than had previously been used have also been carried out.

EXPERIMENTAL

Two series of mice have been treated with solutions of β-propiolactone in acetone (Groups I and II) and one series with 3-bromopropionic acid (Group III). Details of the materials and animals are recorded below.

Materials

β-Propiolactone (Kodak Ltd. or L. Light & Co.) was purified before use by fractional distillation under reduced pressure. The acetone used for Group I was an ordinary grade, purified by fractional distillation only. For Group II "Analar" acetone was boiled under reflux with solid potassium permanganate until no further decolorisation occurred. The solution was filtered, distilled, dried with anhydrous potassium carbonate, filtered again and fractionally distilled with precautions to exclude moisture (Vogel, 1956). Solutions of β-propiolactone were made up 2·5 per cent w/v every 2–3 weeks.

3-Bromopropionic acid (B.D.H.) was applied in a 2·5 per cent w/v solution in the redistilled acetone as used for Group I. All solutions were stored at 4° C.

Animal experiments

The backs of inbred stock albino mice, as normally used in this department for carcinogenicity tests, were shaved before commencing treatment and at intervals thereafter as necessary. Hair growth was appreciably slowed during treatment with β-propiolactone but not with 3-bromopropionic acid.

Group I.—Twenty-five mice were treated with 0·3 ml. of the β-propiolactone solution in redistilled acetone on the back once weekly for 12 weeks, then twice weekly for a further 29 weeks. The 20 surviving mice were killed 5 weeks later. The mice yielded only one tumour, which was identified 41 weeks after commencing treatment as a squamous cell carcinoma.

Group II.—Forty-five mice were treated twice weekly for 60 weeks with 0·3 ml. of the solution of redistilled β-propiolactone in the specially purified acetone. Mice were killed during the experiment as necessitated by the development of tumours. Sections were stained (Ehrlich's haematoxylin and eosin; Weigert's iron haematoxylin and van Gieson's stain) for histological examination by Dr. A. T. Spencer.

Macroscopic papillomas were found in one mouse after only 16 weeks of treatment, and in a further four mice after 20 weeks. Similar tumours appeared on other mice at various times up to 60 weeks, and generally proceeded to squamous cell carcinomas. Althogether 20 mice developed well-differentiated squamous cell carcinomas, showing varying degrees of infiltration of muscle, in the painted

area of skin. In addition, one mouse developed a squamous cell papilloma, one a keratoacanthoma, and two mice showed hyperkeratosis and acanthosis of the skin with changes suggesting early carcinoma. Macroscopic papillomas on five other mice were not examined histologically owing to post-mortem changes. In two mice, killed at 36 and 51 weeks, squamous cell carcinomas developed on the snout while the treated area of skin remained free of tumours. Other findings were two mice with unidentified tumours on the eyelid, one with a spindle cell sarcoma in the painted area of skin, and one mouse with leukaemia. The remaining 11 mice died tumour-free or were killed because of poor condition after 24 to 57 weeks of treatment.

Group III.—Twenty-five mice were treated with the 3-bromopropionic acid solution once weekly for 6 weeks, then twice weekly for 29 weeks. Four mice died during this period, but no evidence of tumour production had been observed when the experiment was terminated 5 weeks later. Since the bromopropionic acid in these experiments was applied in the distilled and not in the specially purified acetone, a check has since been made which has shown that no detectable decomposition, with loss of bromide ion, occurred in this solvent after eleven weeks at room temperature.

Further animal tests

In view of the remarkable resistance of guinea-pigs to most carcinogens of the polycyclic hydrocarbon type, it was thought desirable to see if they would be equally resistant to one of the newer types of carcinogen. β-Propiolactone is therefore being applied twice weekly to shaved guinea-pig skin at concentrations of 2·5 and 5·0 per cent in the purified acetone as used in the mouse experiment (Group II).

One animal, which is white but not a true albino, showed a number of dark patches on the skin after only 16 weeks of treatment with the 5 per cent solution. One patch was removed and identified by Dr. D. J. Parish as a junctional melanoma. This early result is thought to be connected with skin damage which the animal received earlier in the course of fighting. Evidence of tumour production has not been seen on other guinea-pigs which have been treated for 37 weeks.

Action of β-propiolactone on cysteine and cystine

The course of these reactions was conveniently followed using a "Cambridge" Heyrovsky-pattern polarograph with photographic recording as in the albumin experiments already reported (Searle, 1961). For polarography the test solution (1·0 ml.) was added to the ammoniacal cobaltous buffer (10·0 ml.) to which was also added 0·25 ml. of 0·25 per cent gelatin solution. The gelatin suppressed the maximum at the Co^{++}-Co reduction but not the catalytic cysteine maximum, the height of which was measured from the level cobalt diffusion current to give a measure of the amount of unchanged cyst(e)ine in solution.

Cysteine reaction

To 100 mg. of cysteine hydrochloride dissolved in 10 ml. of water and just neutralised to bromothymol blue (pH range 6·0–7·6) was added 0·10 ml. of β-propiolactone (0·115 mg., 2·5 mol. proportions). 0·5 N NaOH was added at

frequent intervals to counteract the acidity produced by hydrolysis of the lactone. After 7·5, 15, 30 and 60 minutes aliquots of solution were removed, diluted 100-fold with water, and polarographed in the cobaltous buffer solution from −0·9 to −2·1 v at a galvanometer sensitivity of 1/200.

For the reactions in weak acid and alkali bromothymol blue was replaced by bromocresol green (pH range 3·6–5·2) and phenolphthalein (8·3–10·0) respectively.

In neutral solution, a trace of cysteine was just detectable 30 minutes after adding the lactone but not after 60 minutes. In mildly alkaline solution all cysteine had disappeared by 7·5 minutes. In the acid range reaction occurred but was very slow, the height of the catalytic cysteine maximum being reduced by only 18 per cent after 30 minutes.

In each experiment the solution was finally made just alkaline to phenolphthalein and warmed to 60°, when some further reaction occurred in the acidic experiment. In no experiment was there any recovery of the catalytic wave, and there is therefore no evidence for formation of thioester in addition to S-2-carboxyethylcysteine.

Cystine reaction

Due to its insolubility cystine solutions containing only 0·76 mg. in 10 ml. were employed, but 0·10 ml. of β-propiolactone were added as before, i.e. a much greater excess. The solutions were added without dilution to the cobaltous buffer, and polarographed at a sensitivity of 1/500.

An unexpected finding was an increase in the height of the catalytic maximum on addition of β-propiolactone. In the experiment in neutral solution the maximum rose from 19·5 scale units at −1·85 v to 24·5 units at −1·94 v after 7·5 minutes, and then rose more slowly to become an inflection at 40 units at −2·1 v after 60 minutes. A slight reduction in height occurred on warming to 60° in alkaline solution but the original maximum was not recovered. The changes observed in the mild alkali were almost identical with those in neutral solution. In mild acid, however, the cystine appeared completely unaffected by β-propiolactone.

Some polarograms obtained in the reactions of β-propiolactone with cystine and cysteine in neutral solution are illustrated in Fig. 2.

DISCUSSION

In the only previous report on the carcinogenicity of β-propiolactone for mouse skin (Roe and Glendenning, 1956) papillomas began to appear on 5 out of 9 surviving mice after 27 weeks of weekly application of the lactone (2·5 per cent in "Analar" acetone). After 40 weeks, tumours on two of these mice became malignant and on two others were regarded as probably malignant. Tumours were obtained earlier in experiments when treatment for the first 5 weeks was with a 5 or 10 per cent β-propiolactone solution, which gave rise to ulceration and scabbing, and scarring appeared to have a tumour-promoting affect.

Our first series of mice treated with β-propiolactone only yielded one tumour. We attributed this result to the decomposition of a considerable proportion of the lactone by water still present in the acetone after fractional distillation. "Analar" acetone, which was used by Roe and Glendenning, is now stated to

contain a maximum of 0·7 per cent of water, which is however sufficient to hydrolyse 2·8 per cent by weight of added β-propiolactone. When purifying this solvent we found it to reduce considerable amounts of potassium permanganate, suggesting the presence of alcoholic impurity which could also react with β-propiolactone. Destruction of lactone would probably be small, however, if the solution were freshly prepared before each application.

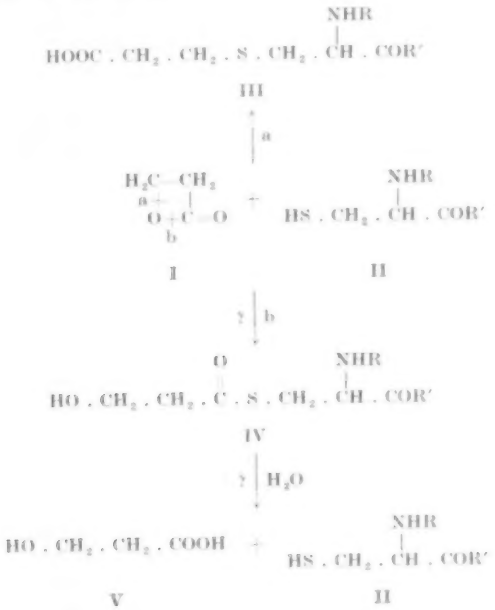

FIG. 2.—Superimposed tracings of polarograms obtained in reactions of β-propiolactone with cystine (A) and cysteine (B) in neutral solution. Polarograms recorded in Brdička's cobaltous buffer containing gelatin, using saturated calomel electrode. (I) Before addition of lactone; (II) 15 minutes and (III) 60 minutes after adding lactone. Galvanometer sensitivity 1/500 (A) or 1/200 (B). (The first wave at approximately 1·0 v represents the Co^{++}–Co reduction.)

When for Group II we used β-propiolactone in permanganate-treated, dried acetone, applied twice as frequently as in Roe and Glendenning's experiments, some papillomas occurred earlier than found by these authors, but on other animals papillomas took as long as 60 weeks to appear. Of the 34 mice of which stained sections were examined 20 had squamous cell carcinomas in the painted area, and of the original 45 mice only 11 were tumour-free at death. A tendency was noticed for tumours to occur somewhat earlier in mice with some skin damage, due to clipping of hair or to fighting, as has been observed with some other carcinogens.

The carcinogenicity of β-propiolactone for mouse skin is thus amply confirmed, though it is of course considerably less than that of the polycyclic hydrocarbon carcinogens which give a high yield of tumours when applied at less than one tenth

of the dosage used here on a weight basis, or about one fortieth of the dose when allowance is made for the much smaller molecular weight of β-propiolactone. It would certainly seem advisable for human exposure to β-propiolactone itself to be as limited as possible.

Whether or not there is any carcinogenic risk attached to materials which have been sterilised with β-propiolactone, however, depends only on its breakdown products since all the lactone is destroyed in the process. Of these the major compounds would be 3-hydroxypropionic acid arising from hydrolysis and 3-chloropropionic acid from reaction with chloride ion, particularly in plasma.

Our first experiment appears to indicate that 3-hydroxypropionic acid is virtually devoid of carcinogenic activity, since in the solution with which these mice were painted the original lactone must have been largely hydrolysed to this acid. Dickens and Jones (1961) have also been unable to demonstrate any carcinogenic activity of hydrolysed β-propiolactone when tested in rats. While 3-chloropropionic acid has not, so far as is known, been tested for carcinogenic activity, the bromo-analogue was found inactive in the experiment reported above (Group III). The only other halogenated fatty acid tested appears to be iodoacetic acid, which Orr (1938) found to cause no changes not attributable to the solvent when he applied it to mouse skin as a 2 per cent solution in benzene.

It is possible that biological material treated with β-propiolactone would contain some S-2-carboxyethylcysteine. This compound was also tested for carcinogenicity by Dickens and Jones, who found a sarcoma in the only rat to survive 87 weeks of repeated injections of 0·5 mg. The significance of this result is difficult to assess at present.

It is difficult to correlate the observed effects of β-propiolactone on cysteine and cystine in protein combination with its action on the free amino acids. This is in part owing to the lack of exact knowledge concerning the origin of the catalytic double wave which is obtained when cyst(e)ine-containing proteins are polarographed in ammoniacal cobaltous buffer. It is usually assumed that only the second part of the double wave is directly due to the presence of cysteine and/or cystine in the protein. If this is so, the results of Searle (1961) indicate that cystine as well as cysteine in the protein can be attacked by β-propiolactone, since similar results were obtained with egg albumin and with bovine serum albumin, only the former of which contains appreciable cysteine in the reduced form. Moreover, the reaction was reversible, leading to the suggestion that a thioester was formed which became hydrolysed once more when the excess of lactone had been destroyed by the solvent.

The experiments with free cysteine, however, gave the results which would be expected in view of Dickens and Jones' isolation of S-2-carboxyethylcysteine in high yield, i.e. a complete irreversible destruction of the cysteine catalytic wave on addition of β-propiolactone. Cystine showed an increase in the catalytic wave due to some interaction which cannot at present be identified.

Some attempts were made during the present work to detect the formation of S-2-carboxyethylcysteine in β-propiolactone-treated albumins by two-dimensional paper chromatography of the desalted acid hydrolysates. This method did not give a definite result since glutamic acid, present in large amount, moved to an adjacent position on the paper with the solvent mixtures employed. It was confirmed that little if any destruction of S-2-carboxyethylcysteine would have occurred during hydrolysis.

We have thus no evidence that S-alkylation of cysteine can occur when the amino acid is in protein combination, or whether the ready S-alkylation of free cysteine has significance with regard to carcinogenesis.

SUMMARY

1. β-Propiolactone gave rise to papillomas and squamous cell carcinomas in a high proportion of mice when applied to the skin in carefully purified acetone.

2. 3-Bromopropionic acid showed no evidence of carcinogenicity for mouse skin.

3. The rapid reaction of β-propiolactone with cysteine, followed polarographically, was accelerated in mild alkali and retarded in mild acid.

4. Some form of interaction with cystine, also accelerated by mild alkali, was suggested by a rise in the polarographic catalytic maximum in cobaltous buffer.

5. Unlike the reaction of β-propiolactone with albumin cyst(e)ine groups, the changes observed with free cystine and cysteine were irreversible.

I am very grateful to Dr. A. T. Spencer for reporting on the tumour sections, and to Dr. D. L. Woodhouse for helpful discussions. The work was supported by the Birmingham Branch of the British Empire Cancer Campaign.

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LIVER CHANGES AND PRIMARY LIVER TUMOURS IN RATS GIVEN TOXIC GUINEA PIG DIET (M.R.C. DIET 18)

REGINA SCHOENTAL

From the Toxicology Research Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey

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DURING recent years, outbreaks of oedema and liver damage among guinea-pigs have been reported from several laboratories (Paget, 1954 ; Stalker and McLean, 1957), and have been traced to feeding with certain batches of the commercial pelleted Diet 18. This diet, devised by Bruce and Parkes (1947), should contain the following ingredients :—

Dried grass meal	30 per cent
Barley meal	20 per cent
Bran	15 per cent
Ground nut cake	15 per cent
Linseed cake	10 per cent
Dried meat and bone	8 per cent
Calcium carbonate	1 per cent
Sodium chloride	1 per cent

According to Stalker and McLean (1957) the millers, facing difficulties in obtaining dried grass meal from the original sources, found new suppliers. It was therefore suspected that this ingredient may be responsible for liver damage in the guinea-pigs. Dried grass collected in Great Britain may be contaminated with some common weeds of the genus *Senecio* (ragwort, groundsel), while imported batches may contain other weeds of the genera *Heliotropium*, *Crotalaria*, etc. some species of which are known to contain hepatotoxic pyrrolizidine (*Senecio*) alkaloids (Warren, 1955). Stalker and McLean (1957) report that they actually tested chloroform extracts of the suspected diet in guinea-pigs, but could not reproduce the toxic effects of the faulty diet. Further tests reported by these workers disclosed a high content of titanium, iron and lead in this diet, but they concluded that these elements could not be responsible for the pathological findings in the affected animals.

Pyrrolizidine alkaloids are often present in the plants in the form of their N-oxides which may constitute the major part of the alkaloidal content (Koekemoer and Warren, 1951). The N-oxides are not readily extractable with chloroform but will remain in the watery phase, unless reduced to the alkaloids prior to the chloroform extraction. N-oxides are, however, as hepatotoxic as their parent alkaloids (Schoental, 1955).

In their report, Stalker and McLean (1957) did not state how much alkaloid was present in their chloroform extracts given to the experimental guinea-pigs, but the amounts might only have represented a fraction of the alkaloidal sub-

stances present in the diet. As pyrrolizidine alkaloids when given below the critical dosage may not show any effects, the results of Stalker and McLean did not exclude the possiblity that pyrrolizidine alkaloid-containing plants might have been the cause of the trouble.

A similar outbreak of liver damage in guinea-pigs occurred at the Agricultural Research Council Institute of Animal Physiology in Babraham. In October 1957, Dr. E. J. H. Ford sent us 112 lb. of the suspected, pelleted, Diet 18, and a simple " biological " test was performed.

Weanling male Wistar rats, 40–45 g. body weight, of the Porton strain, were used. Five animals were given the suspected diet, five others Diet 18, from batches which had not caused any untoward effects in our rabbits and guinea-pigs. Both groups were given food and water *ad libitum*.

In both groups, the growth rate of the young rats was similar, but it was much below that of rats given MRC Diet 41, devised for rats (Bruce and Parkes, 1949). In the control group on " normal " Diet 18, one rat died after six weeks from bronchopneumonia and two were killed when ill after 10 months. Of these one had severe kidney and bladder damage due to calculi, the second had congestion of the lung.

The supply of the suspected diet became exhausted after 12 months; the surviving rats, two in the control group and five in the experimental one were then killed. The livers of all those rats on the suspected diet were rather enlarged, comprising 5–10 per cent of body weight. In three there were a few small nodules, some translucent and others grey or dark. The remaining two rats had pronounced nodular livers, one of which was particularly large (10 per cent body weight) and studded with various sized nodules (Fig. 1). Other organs of these rats did not show gross abnormalities.

Microscopically, the translucent nodules show gross cystic hyperplasia of the bile ducts and these livers contain also hyperplastic nodules and some areas of fatty change. Parenchymal cells vary in size and are often greatly enlarged. The bigger of the nodules in the liver shown in Fig. 1 prove to be liver cell carcinomas (Fig. 2 and 3).

The livers of the rats in the control group appeared slightly granular, and were of the normal size, 4–4·5 per cent body weight. Microscopically there is slight infiltration in the portal spaces with small cells, areas of fatty change and some variation in the size of the parenchymal cells.

The lesions in the livers of the experimental group of rats were very similar to those seen in rats treated with pyrrolizidine alkaloids (Schoental, Head and Peacock, 1954; Schoental and Magee, 1957, 1959).

However, a hepatotoxic agent cannot be identified by the microscopic features of the liver lesions which it produces any more than can an agent which causes tissue inflammation. Alcoholic extracts of the toxic guinea-pig diet were therefore prepared and processed in the usual way for chemical testing for pyrrolizidine alkaloids and their N-oxides. No definite evidence was obtained for the presence of alkaloidal substances in these abstracts.

Several dried grass samples were then obtained from various dealers and tested in rats for hepatotoxic action and chemically for the presence of alkaloidal constituents. All these tests gave essentially negative results. Thus, the carcinogenic factor responsible for the primary liver tumours induced in our rats by the toxic guinea-pig diet remained unidentified.

Recently new evidence from a different side clarified the problem. Outbreaks of fatal liver disease among turkeys (Blount, 1961), ducklings and poultry (Allcroft *et al.*, 1961 ; Carnaghan and Sargeant, 1961) cattle and pigs (Loosmore and Markson, 1961) have been traced to ground nut meals, some batches of which contain a hepatotoxic agent. The chemical nature of this agent is not yet established, but it appears not to be alkaloidal (Allcroft *et al.*, 1961). The toxic ground nut meals added to Diet 41 have been found to induce in rats primary liver tumours indistinguishable from those caused by the toxic guinea-pig diet (Barnes and Magee, personal communication ; Lancaster, 1961). Furthermore, when this ground nut meal was added to a non-toxic Diet 18, young guinea-pigs died within 2–3 weeks with ascites, and tissue oedema accompanied by early liver damage (Barnes and Magee, personal communication). The diet used in the experiments described above, and carried out in 1958, contained 15 per cent of ground nut cake. The results obtained are compatible with the assumption that a toxic ground nut meal was used in the preparation of this batch of Diet 18.

I am indebted to Dr. E. J. H. Ford, Babraham, for the supply of toxic Diet 18, Dr. W. Lane-Petter for the provision of the grass samples, Professor W. Orr and Dr. P. N. Magee for the evaluation of the liver lesions and Dr. J. M. Barnes for his interest in this work. I wish also to thank Mr. R. F. Legg for the photomicrographs and Mr. M. R. Greenwood for valuable technical assistance.

SUMMARY

A batch of MRC Diet 18 which has been found to be toxic to guinea-pigs at the Agricultural Research Council Institute of Animal Physiology, Babraham, was tested chemically for the presence of pyrrolizidine alkaloids or their N-oxides and by feeding to rats. No alkaloidal constituents were detected in this diet, which induced, however, liver lesions in rats similar to those following treatment with pyrrolizidine alkaloids. The toxicity of this diet is likely to be due to its content of ground nut meal (15 per cent) some batches of which have been recently reported to cause similar toxic lesions in guinea-pigs and liver tumours in rats.

Addendum

The toxic factor in the ground nut meal has been traced to a metabolite of *Aspergillus flavus* with which some batches of ground nuts are contaminated (Sergeant *et al.*, 1961 ; Lancaster *et al.*, 1961).

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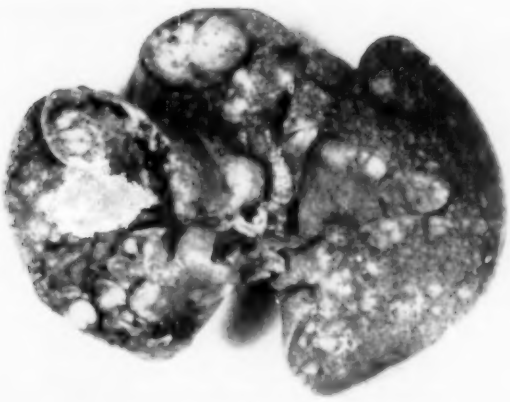
EXPLANATION OF PLATE

Fig. 1.—Male rat, kept on toxic guinea-pig diet for 12 months. Greatly enlarged liver, 10 per cent of body weight, hyperplastic and neoplastic nodules ×0·9.

Fig. 2.—Same liver as in Fig. 1. Part showing liver cell carcinoma. H. and E. ×100.

Fig. 3.—Same liver as in Fig. 1 showing liver cell carcinoma under higher magnification. H. and E. × 400.



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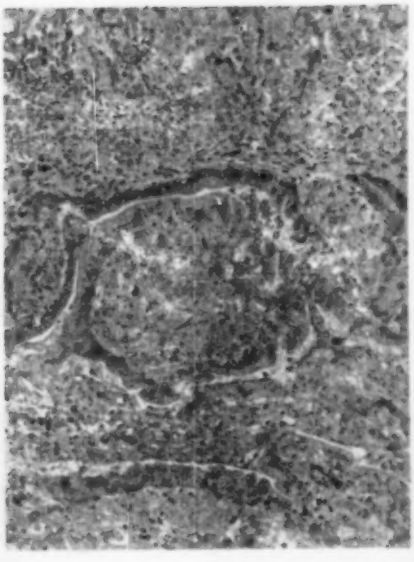

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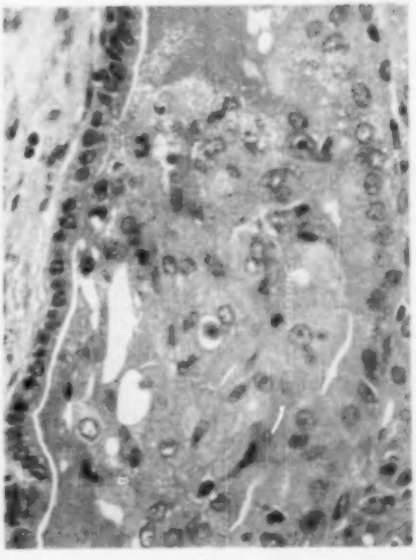

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THE SELECTION OF GASTRIC CARCINOGENS

FRANCIS E. RAY, MARY ANN CROMER,
ARTHUR C. AYCOCK AND NELLIE PITZER

From the Cancer Research Laboratory, Department of Pharmaceutical Chemistry, University of Florida, Gainesville, Florida, U.S.A.

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IT has long been recognized that a reliable method for the production of adenocarcinoma of the animal stomach would greatly facilitate research into the etiology, diagnosis and therapy of this form of cancer. The poor prognosis of gastric cancer in man makes these studies highly desirable. The difficulties in producing carcinoma of the glandular stomach in animals has been described in several comprehensive papers that have appeared during the past two decades (Beck, 1946; Klein and Palmer, 1941; Stewart, Snell and Hare, 1958), as well as in many others.

Until the work of Morris, Wagner, Ray, Snell and Stewart (1961) no method for the experimental induction of adenocarcinoma of the animal stomach by oral administration of carcinogens had been successful.

It appeared reasonable to the early workers that a potent epithelial carcinogen such as methylcholanthrene or benzopyrene would, on administration *per os*, attack the gastric mucosa and produce stomach cancer. Only squamous forestomach tumors, however, were obtained. Ray and Jung (1951) reasoned that because the glandular tissue was protected against contact carcinogenesis by the flow of mucus and other secretions, no effective attack could take place. Efforts to penetrate or destroy the mucus barrier by Setälä and Ermala (1951) and by Richardson (1956) yielded doubtful results. It seemed to us that if we were to be successful we would have to find a carcinogen that would be absorbed into the circulation and be secreted into the lumen of the stomach. In passage it would come in intimate contact with the immature glandular cells which should be most susceptible to attack (Ray, Cambel, Jung, Peters and Woislawski, 1953).

The work on gastric secretion in the dog by Dawson and Ivy, (1925) and by Ingraham and Visscher (1935) showed that basic dyes appear in considerable concentration in the stomach when injected intravenously. We were able to extend this observation to rats (Ray and Peters, 1951) and to the intraperitoneal route of administration (Cambel, Breidenbach and Ray, 1954).

If the basicity of a compound facilitates its secretion into the stomach, the ionization constant (pK'_a) should be related to the extent of secretion (Ray and Jung, 1951). This, within certain limitations, proved to be true (Ray and Pease, 1957). One limitation was that the dye must not be metabolized to a substance of different basicity before its passage through the stomach wall. It is possible that the diffusion coefficient of the substance may also be of significance.

Our choice of a basic carcinogen first fell on 2-aminofluorene, since it seemed to be the most versatile basic carcinogen known. Using the radioactive derivative

of Ray and Geiser (1950; cf. 1949), Weisburger, Weisburger and Morris (1951) showed that when acetylated aminofluorene was administered to the rat it was rapidly deacetylated. Other work by Morris, Dubnik and Johnson (1950) showed that aminofluorene and its N-acetyl derivative were equally carcinogenic. It seemed possible, nevertheless, that a variation of N-acyl groups, and other substituents, might influence the degree of carcinogenicity. This proved to be the case (Morris, Velat, Wagner, Dahlgard and Ray, 1960) and it was possible, to a certain extent, to relate the degree of carcinogenicity to the ease of hydrolysis of the N-acyl group (Argus and Ray, 1959).

Not only was the degree of carcinogenicity altered, but the site of attack was often shifted. For example, using N-2-fluorenylsuccinamic acid, it was found that carcinogenic attack on the mammary gland was almost completely eliminated in female rats, while a substantial number of liver tumours developed (Morris, Velat, Wagner, Dahlgard and Ray, 1960). The N-2-fluorenylphthalamic acid of Argus and Ray (1959) gave rise to the Morris hepatoma 5123 (Morris and Wagner, 1961), which varied considerably in enzymatic activity from most other hepatomas. Unfortunately, none of the many sites attacked by 2-aminofluorene derivatives was the glandular stomach.

On re-examining the evidence, it seemed that the reason for failure lay in the fact that aminofluorene was not basic enough. It was decided to increase the basicity by adding *another* amino group to produce 2, 7-diacetylaminofluorene (also called N, N′-2, 7-fluorenylenebisacetamide). The ionization constants of 2-aminofluorene and 2, 7-diaminofluorene were measured recently by Grantham, Weisburger and Weisburger (1961). In 70 per cent ethanol 2-aminofluorene has a pK'_a of 4·31, while 2, 7-diaminofluorene, which has two basic groups, gives pK'_a 4·97, and pK''_a 3·5. Thus, as expected, 2, 7-diaminofluorene is considerably more basic than 2-aminofluorene.

When fed to 47 Buffalo and AXC strain rats for a maximum of 47 weeks, 2, 7-diacetylaminofluorene produced four carcinomas of the glandular stomach (among many other tumours), one in the fundus and three in the pyloric region (Morris, Wagner, Ray, Snell and Stewart, 1961). The effect may have been due to the greater carcinogenicity of the 2, 7-compound which was evident by greater production of lung, submaxillary gland, intestinal, uterine and auditory canal tumours. There was also a larger number of leukemias. On the other hand, more mammary gland tumours were obtained with the 2-amino compound, as well as an equal or slightly greater number of liver tumours. It was of interest, then, to determine if the more basic 2, 7-diamino compound is indeed secreted more readily than the 2-amino.

MATERIALS AND METHODS

Carcinogens

One gram each of 2-nitrofluorene and 2, 7-dinitrofluorene was treated with 15 curies of tritium gas at 27° and 0·39 atmosphere for two weeks. The specific activity of the mononitro compound was 0·51 mc/mg. The specific activity of the 2, 7-dinitrofluorene was 0·95 mc/mg. The substances were reduced catalytically with hydrogen and platinum, acetylated and recrystallized until the mother liquor showed a constant activity. The analysis was by the method of Wilzbach, Van Dyken and Kaplan (1954) using an ionization chamber and Cary No. 31 vibrating reed electrometer. By carrying out two reactions on the material it

was possible to eliminate radioactive impurities. The radiation of amines tends to produce large amounts of impurities that are difficult to separate. The nitro compounds, however, are comparatively stable. The resulting acetylated amines had rather low specific activities of 31·04 μc/mg. for 2-acetylaminofluorene and 21·98 μc/mg. for 2, 7-diacetylaminofluorene. This indicates that the major amount of tritium activity had been concentrated at the reactive methylene group in the 9-position. On reduction of the nitro groups this was exchanged for hydrogen. It is of interest to note that before reduction the dinitro compound had the greater activity. This agrees with the known greater activity of the methylene group in disubstituted fluorenes. In addition, 2, 7-dinitrofluorene has one less nuclear hydrogen for permanent replacement by tritium.

Animals

Six male Sprague-Dawley rats weighing 450–550 g. were fasted for a 24 hr. period, followed by intraperitoneal injections of Nembutal (15 mg./kg.). Further anesthesia was induced by ether inhalation. The abdominal hair was shaved, the skin swabbed with 70 per cent alcohol, and a 3-cm. mid-line incision made at the level of the stomach. The gastro-intestinal tract immediately below the pylorus was ligated to retain the secreted materials within the stomach. After closing the incision the animals were allowed a 2-hr. rest period. They were then divided into two groups, one receiving 4·5 mg. 139·7 μc of labeled 2-acetylaminofluorene in one ml. of 1 per cent methyl cellulose, the other, 4·5 mg. (98·9 μc) of labeled 2, 7-acetylaminofluorene per animal intraperitoneally. Four hours later the animals were sacrificed, the esophagus ligated at its lower end, the stomach and its contents were excised. The stomach and contents were separately mixed with a solution of 1 per cent sodium hydroxide, 10 ml./g. and allowed to stand for two days in the cold room. Following this the samples were autoclaved for 30 minutes at 250° F at 18 lb./sq. in.

Individual samples, using 0·1 ml. of the stomach and its contents, were heated in a sealed tube with 0·2 g. of nickel and 1 g. of zinc at 700° C. for 3 hr. in an electric furnace. These samples were transferred to an ionization chamber for analysis. The amount of radioactivity was determined separately in the stomach and in the contents.

RESULTS AND DISCUSSION

The data show (Table I) that 2, 7-diacetylaminofluorene is taken up by the stomach wall to a greater extent (141 per cent) than is 2-acetylaminofluorene. On

TABLE I.—*Radioactivity in Stomach Tissue and Contents**

	2-Acetylaminofluorene-H^3		2, 7-Diacetylaminofluorene-H^3		Ratios 2,7-/2-
	μg.	%	μg.	%	
Stomach wall§	33·82/4500†	0·752	47·77/4500†	1·062	1·061/0·752=1·41
Stomach contents	30·22/4500†	0·673	25·02/4500†	0·556	0·556/0·673=0·83
					$\frac{\text{R SW}}{\text{R SC}}$‡ = 1·70

* Data in total μg. and per cent of dose per average animal.
† Total amount found/original dose.
‡ Ratio of the two ratios, 1·41/0·83 : stomach wall/stomach contents.
§ Average weight 3·5 g. Three animals used for each compound.

the other hand, only 83 per cent as much 2, 7- is found in the stomach contents. The ratio between these two percentages is 170 per cent, which seems large enough to indicate a real difference in the behaviour of the two compounds.

Preliminary experiments some years ago convinced us that 2-acetylaminofluorene was, indeed, secreted by the stomach of the rat (Ray, Cambel, Jung, Peters and Woislawski, 1953) so this was no surprise. It was surprising, however, to find the amount to be comparatively so great. Obviously, it is not the degree of secretion that is important. It is probable that the proportion retained in the stomach wall is the critical factor. Since, in carcinogenesis, these compounds are fed for a prolonged period, it is possible that a considerable differential build-up of the 2, 7- compound takes place. It has been well demonstrated by Weisburger and Weisburger (1954) and Dyer and Morris (1956), and others, that protein binding occurs between 2-acetylaminofluorene and the tissues of rats. Since this seems to be a necessary first step in carcinogenesis it may well be that the 41 per cent increase in the stomach tissue added to the somewhat greater potency of 2,7-diacetylaminofluorene was just sufficient to exceed the critical stage. It is, of course, conceivable that the amount of 2, 7-diacetylaminofluorene localized in the stomach wall is not sufficiently greater to be a deciding factor : the difference then would be attributed solely to the increased carcinogenicity of the 2, 7- compound. In either case the basicity of the compound seems to be a necessary prerequisite and points the direction towards further investigation.

SUMMARY AND CONCLUSION

The secretion of 2-acetylaminofluorene and 2, 7-diacetylaminofluorene by the stomach has been compared. The latter alone causes adenocarcinoma of the stomach and 41 per cent more of the administered dose was found in the stomach wall ; some 17 per cent more of the former was found in the contents. Over a period of time the increased percentage taken up might result in a large differential in the stomach tissue. On the other hand, the relative amount taken up may not be significant and the carcinogenicity may be attributed entirely to the greater potency of 2, 7-diacetylaminofluorene. The basic nature of the compounds in any event enables them to reach the target area.

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THE EFFECT OF HYPOPHYSECTOMY ON THE DEVELOPMENT OF OVARIAN TUMOURS IN MICE TREATED WITH DIMETHYLBENZANTHRACENE

JUNE MARCHANT

From the Cancer Research Laboratories, Department of Pathology, Medical School, Birmingham, 15

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OVARIAN tumours may be induced in rats and mice by at least three techniques. One method, used most commonly in rats, is by transplantation of an ovary to the spleen of a castrated animal or to some other site drained by the liver. This method gave rise to a hormonal theory of ovarian tumour induction put forward by Biskind and Biskind (1944). The oestrogens produced by this transplanted ovary drained to the liver, where they were destroyed. The consequent lack of circulating oestrogen led to an increase in pituitary gonadotrophin production, which in turn acted on the transplanted ovaries and resulted in the growth of tumours in them. Intact ovarian function was found to inhibit ovarian tumour production in intrasplenic ovaries (Biskind and Biskind, 1948), presumably by controlling gonadotrophin production.

The other two most commonly used methods of ovarian tumour induction are by giving sterilising doses of X-rays (Furth and Butterworth, 1936), or by administering to mice certain chemical carcinogens, of which the most effective seems to be 7, 12-dimethylbenz(α)anthracene, or DMBA (Howell, Marchant and Orr, 1954). Since intact ovarian function can inhibit tumour production by X-rays (Kaplan, 1950) and by DMBA (Marchant, 1960*a*), it is believed that the pituitary hormonal mechanism is also involved in these methods of ovarian tumorigenesis.

However, experiments indicate the possibility that some factors other than mere elevation of gonadotrophins acting on ovarian tissue may normally be concerned in ovarian tumour induction. Parabiosis of intact animals castrated (in order to secure elevated gonadotrophins in the intact partner) rarely gives rise to ovarian tumours alone, but causes elevation of tumour incidence when combined with chemical carcinogen treatment (Bielschowsky and Hall, 1951) or with intrasplenic ovarian grafting (Mühlbock, 1953). Also, administration of sufficient testosterone to prevent post-castrational elevation of gonadotrophin levels failed to prevent ovarian tumours developing in irradiated mice (Gardner, 1950; Chang and van Eck, 1952).

The present experiments represent initial attempts to distinguish the relative contributions to ovarian tumour production made by DMBA and by pituitary hormones. It is convenient to separate ovarian tumorigenesis by DMBA into at least 2 phases. There is a "preneoplastic" phase involving destruction of follicles and oocytes resulting in premature senility of the ovaries. In mice of the genetic constitution used herein, it is known that follicular destruction is

almost complete in about 3 months from commencing fortnightly treatment with DMBA (Marchant, 1959*a*). Further degeneration and atrophy continue to occur, but from this time onwards a second phase may, or may not, follow. This is the appearance and growth of tumours of the granulosa-celled type, in numbers steadily increasing with time.

The experiments described below were done to see how the absence of pituitary hormones would affect, first, the whole process of ovarian carcinogenesis by DMBA and, second, affect (*a*) the induction of the preneoplastic phase by DMBA and (*b*) the appearance and growth of tumours from preneoplastic ovaries.

MATERIALS, METHODS AND RESULTS

Mice used in these experiments were first generation hybrid females derived from C57Bl mothers and IF fathers. They were housed five to a box and fed on rat cubes, known as the Thompson diet (Heygate and Sons) with water *ad libitum*. The carcinogen treatment consisted of fortnightly skin paintings of 0·5 ml. 0·5 per cent DMBA in olive oil (2·5 mg.).

Hypophysectomy was performed when the mice weighed between 13 and 20 g. They were given 1 per cent glucose-saline to drink for the first 2 days and were kept at a constant temperature of 80° Fahrenheit thereafter. Hypophysectomised mice were weighed once a week.

Orthotopic ovarian grafting was done using the technique described by Jones and Krohn (1960). Ovaries were weighed at the time of removal or transplantation. Daily vaginal smears were taken from all mice over lengthy periods of their lives.

At necropsy the condition of ovaries was noted and these were fixed in formol-saline. Sections representative of 3 or 4 different levels through the ovary were examined histologically. Whole-mount preparations of breast tissue were made from some of the mice.

1. *The effect of DMBA-treatment of hypophysectomised mice*

In this experiment mice of 15 to 20 g. were hypophysectomised. Three to 4 weeks later, fortnightly paintings of DMBA were begun.

Results.—After the second fortnightly painting, the animals appeared sick and 9 of 11 died within 2·5 to 5 weeks from the first painting. The cause of death was not ascertained, but it is believed that death was due to the acute toxic effects of DMBA (Shubik and Della Porta, 1957). There was a weight loss of 2·5 to 7·5 g. All ovaries were very small, weighing about 1 mg. Histologically they were composed of follicle remnants and a small number of follicles with oocytes and granulosa cells, together with theca and stroma. Mature follicles and corpora lutea were absent (Fig. 3). Vaginal smears were anoestrus.

Owing to the toxic effects of DMBA on hypophysectomised mice, it was decided to make use of transplantation methods to study the effect of hypophysectomy on the separate phases of ovarian carcinogenesis by DMBA.

2(a) *The effect of hypophysectomy on the induction of preneoplastic changes in ovaries by DMBA*

It is known that DMBA-treatment of normal mice will render their ovaries preneoplastic, so that when subsequently transplanted orthotopically into normal

mice they will grow into tumours in a high proportion of cases after about 15 months (Marchant, 1959*b*). It has since been found that the amount of DMBA treatment (from 1 to 6 paintings) has little effect on the final tumour yield. Ovaries grafted from untreated mice do not become tumorous (Marchant, 1960*b*). In the present experiment it was decided to see whether the absence of a pituitary would prevent DMBA-treatment from rendering ovaries preneoplastic, so that they would be unable to grow into tumours on subsequent transplantation to normal hosts.

Donors.—Hypophysectomised mice were painted twice with DMBA, as in experiment 1. Four weeks from the first painting the ovaries of 14 survivors were removed and grafted orthotopically to untreated mice. At the time of grafting, 13 of the mice had smooth, yellow ovaries weighing 1 mg. or less and anoestrus vaginal smears (Fig. 3). The fourteenth was judged incompletely hypophysectomised for, at the time of grafting, its ovaries were pink with white spots and vaginal smears showed oestrus cycles. This mouse lived for 56 weeks and eventually more than doubled its weight at "hypophysectomy". Of the other 13 mice, 6 died 4 to 7 weeks from the first DMBA treatment with a weight loss of 2 to 5 g. The other 7 survived the initial toxic effects of DMBA and lived for 18 to 49 weeks from the first treatment, without gain in weight.

Hosts.—The ovaries from the hypophysectomised DMBA-treated donors were grafted into the ovarian capsules of 13 normal mice 8 or 9 weeks old whose own ovaries were removed. The weight of the hosts' own ovaries at removal was between 2·5 and 7·5 (mean 4·2) mg.

The animals were all killed 15 months after the grafting operation.

Results.—Four of the 13 mice showed no evidence of ovarian tumours. One of these four had an ovarian cyst 5 mm. in diameter, filled with clear fluid, but the ovaries of the other three were about 2 mm. in diameter, composed of large cells filled with ceroid pigment and, in two cases, some "anovular follicles" or "sterile tubules". Their uteri were normal-sized or thin and vaginal smears were anoestrus at the time of death. Breast ducts showed varying degrees of atrophy and there were no acini present.

Three mice had ovaries unequal in size, the bigger being not larger than a normal ovary. These were found to contain small tumour nodules, being otherwise similar to the ovaries of the four mice just described. Their uteri were normal-sized to plump and vaginal smears showed some evidence of suboestrus activity. Breast ducts were variable—fine, normal or dilated—and there were end-buds, terminal acini or acinar clusters.

The other 6 mice had unilateral granulosa-cells ovarian tumours ranging from 4 mm. to 15 mm. in diameter (Figs. 4 to 6). Three of them had cysts filled with clear fluid up to 6 mm. diameter in the contralateral ovary. Uteri were variable, with a tendency to cystic hyperplasia. Vaginal smears were also variable, but in 4 of the 6 animals there was evidence of oestrus or suboestrus activity. Breast acini were absent from 2 mice and variable in the other 4.

There seemed to be no definite correlation between the condition of ovaries, uterus and breast tissue, but a general tendency for the mice with larger ovarian tumours to show more cystic hyperplasia of the uterus, evidence of oestrus activity from vaginal smears and greater development of breast acini.

The ovaries from the fourteenth (incompletely hypophysectomised) donor were also grafted into a normal mouse and one grew into a dark red tumour

17 mm. in diameter, weighing about 3 g. It proved to be a pseudofollicular granulosa-cell tumour with cystic spaces, some of which were filled with blood. The uterus was distended with fluid, vaginal smears showed persistent oestrus and breast tissue showed clusters of acini.

2(b) *The effect of hypophysectomy on the development of tumours from ovaries rendered preneoplastic by DMBA*

It was decided to see whether ovaries from mice which had been treated with DMBA for 3 months would be able to grow into tumours when transplanted orthotopically into hypophysectomised hosts. It is known that a small proportion of such ovaries may be expected to contain incipient tumours (Marchant, 1959*a*) and that they would be expected to grow into tumours in a high proportion of cases within 15 months of grafting orthotopically to hosts with intact pituitaries. (Marchant, 1959*b*).

Donors.—In this experiment 21 mice aged from 3 to 4 months were given 6 fortnightly paintings of DMBA. Three months from the first painting their ovaries were removed, at which time they were fairly smooth and yellowish and the mean weight of 41 ovaries was 3·8 mg. (range 2·0 to 6·5) see Fig. 8. The remaining ovary was cystic with haemorrhage into the lumen and weighed 15 mg.

EXPLANATION OF PLATES

Figures show sections of ovaries from F_1 C57Bl × IF mice stained with haematoxylin and eosin. All are shown at the same magnification (× 27) except Fig. 6.

Fig. 1 and 3 show the follicular destruction caused by the action of DMBA on hypophysectomised mice.

Fig. 1.—Normal mouse weighing 15 g. Ovary, weighing about 1·5 mg., showed many follicles in various stages of maturity and atresia together with some young corpora lutea.

Fig. 2.—Young mouse hypophysectomised 4 weeks previously. Ovary, weighing about 1 mg., showed many small follicles, but no mature ones or corpora lutea.

Fig. 3.—Similar hypophysectomised mouse which subsequently received 2 fortnightly DMBA paintings. Ovary, weighing less than 1 mg., showed much follicular destruction and few normal follicles remained 4 weeks after the first DMBA painting.

Fig. 4 to 6 show parts of ovarian tumours which developed 15 months after orthotopic grafting of ovaries similar to Fig. 3 into hosts with intact pituitaries. The ovaries containing tumours ranged in weight from 7 mg. to 3 g.

Fig. 4.—Two nodules of undifferentiated granulosa-cells tumour which arose in a diffusely luteinised ovary weighing about 15 mg.

Fig. 5.—Section through the edge of a granulosa-celled tumour weighing about 1 g.

Fig. 6.—High-powered view of part of Fig. 5 showing, in the lower part, the luteinisation of the cells of the tumour. × 112.

Fig. 7 and 8 show the follicular destruction caused by the action of DMBA on intact mice.

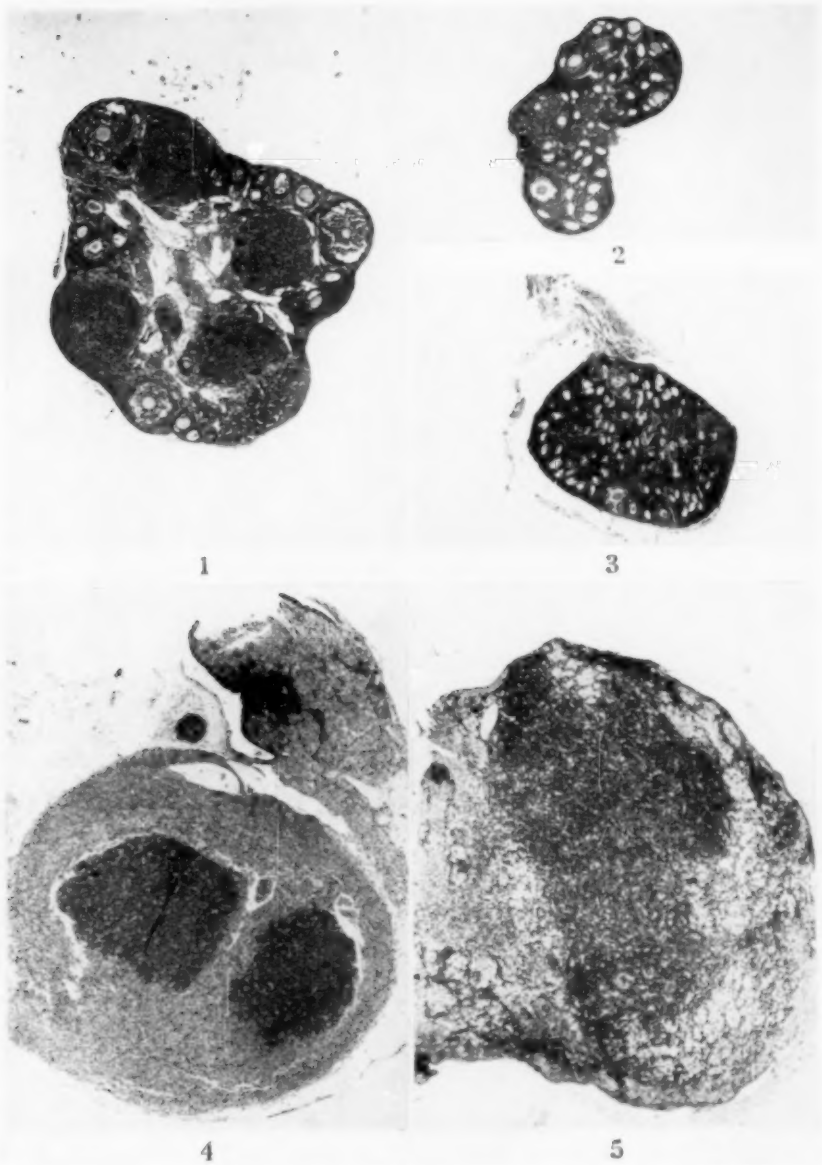
Fig. 7.—Normal mouse aged 6 months. Ovary, weighing 8·5 mg., showed follicles in various stages of maturity and atresia and many corpora lutea of different ages.

Fig. 8.—Mouse aged 6 months which had received 6 fortnightly paintings of DMBA. Preneoplastic ovary, weighing about 4 mg., showed normal follicles had disappeared. Atretic follicles were still seen. Corpora lutea were merging. (Such ovaries would develop into tumours in hosts with intact pituitaries.)

Fig. 9 and 10 show the atrophic fate of preneoplastic ovaries similar to Fig. 8, 17 months after orthotopic grafting into hypophysectomised hosts.

Fig. 9.—This picture was typical of the majority of such grafts. The ovaries were represented by a small cluster of pigmented cells with, or without, a few anovular follicles.

Fig. 10.—A small haemorrhagic cyst such as was associated with the remnants of a few grafts of preneoplastic ovaries in hypophysectomised hosts.



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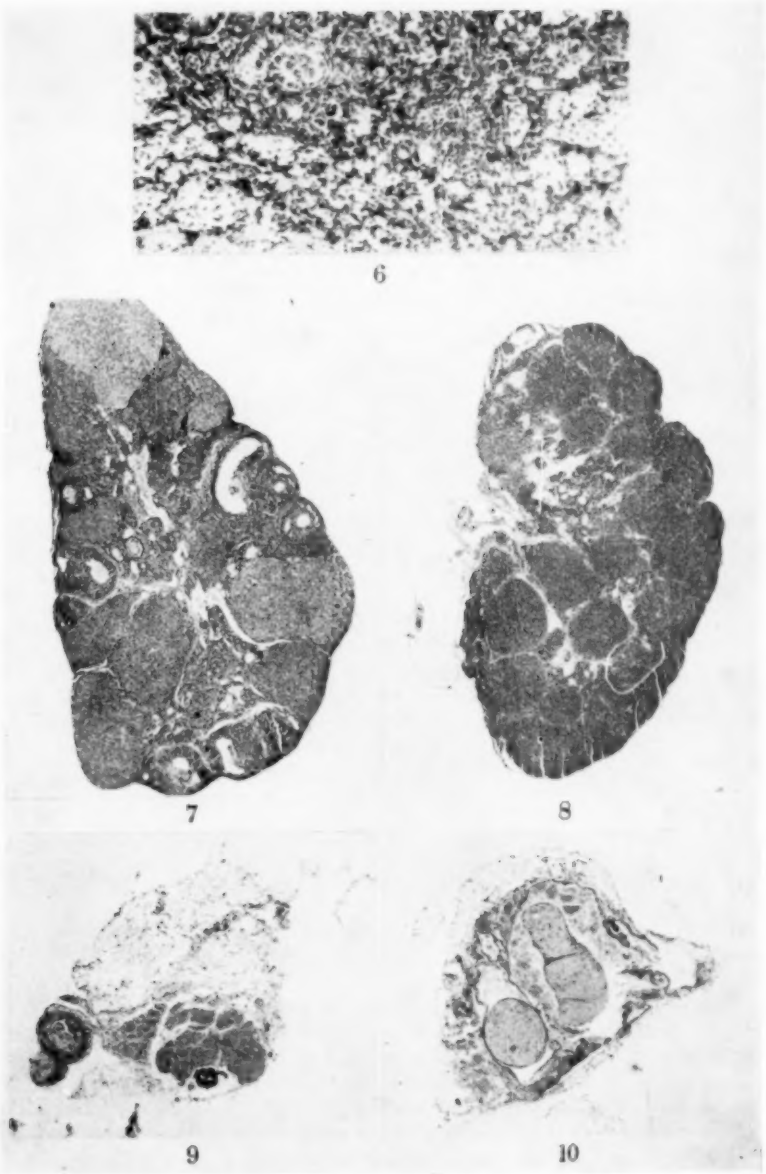

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Hosts.—The ovaries from the DMBA-treated mice above were grafted into the ovarian capsules of young mice which had been hypophysectomised one month earlier. The hosts own ovaries, which were removed, weighed about 1 mg. and were full of follicles and oocytes of all sizes except mature ones. No corpora lutea were present (Fig. 2).

Results.—Of the 21 host mice, 13 survived for 16 months after ovarian grafting, when they were killed and examined for ovarian tumours. The body weights of 4 animals remained static, but in the other 9 there was a gradual increase in weight, commencing about 2 or 3 months after hypophysectomy, indicating probable regeneration of pituitary remnants. Vaginal smears were anoestrus in all but 2 cases and uteri were thread-like. Breast tissue of all animals examined was very atrophic, consisting of very thin ducts branching over a very small area.

Despite the evidence for some pituitary regeneration, no incipient granulosa celled tumours were found in any of the animals of this group. The majority of the ovaries of the 13 surviving hosts were about 1 mm. in diameter, yellow and composed of pigmented cells (Fig. 9) and sometimes anovular follicles in addition. Cysts up to 8 mm. diameter were found in 7 ovaries (Fig. 10) and 3 of them contained much blood. In one animal, with increased body weight and long periods of oestrus, the ovaries weighed 6·5 and 7·5 mg. respectively. They were packed with pigmented cells and there were many small follicles with oocytes around the periphery but no corpora lutea.

DISCUSSION

It is evident, from experiment 1 described above, that hypophysectomised mice are extremely sensitive to the toxic effects of DMBA treatment. A similar effect has been found in hypophysectomised rats by J. S. Howell (unpublished). It was, therefore, not possible to see how the absence of pituitary hormones would affect the complete process of ovarian tumorigenesis by DMBA. However, from the experiments described herein, and others, it is possible to build up some kind of picture of the relative contributions to ovarian tumorigenesis by DMBA and pituitary hormones.

Ovaries of normal F_1 C57Bl × IF mice of about 15 g. body weight weigh about 1·5 mg. They contain many oocytes and follicles in various stages of maturity and atresia and a number of young corpora lutea (Fig. 1). When such mice are hypophysectomised, they fail to develop any more corpora lutea, and existing ones appear to have been resolved one month later. Atretic follicles are present and there are numerous small follicles with granulosa cells but no mature follicles (Fig. 2). There is consequent loss of weight of the ovary. Not only does hypophysectomy prevent the maturation of ovarian follicles and thereby prevent formation of corpora lutea, but it also significantly reduces the normal rate of oocyte loss due to atresia (Jones and Krohn, 1961). However, treatment of hypophysectomised mice with DMBA results in considerable destruction of oocytes and follicles, as shown in Fig. 3, one month later. Experiment 2 showed that such ovaries are capable of growing into tumours, as seen in Figs. 4 to 6, when transplanted orthotopically to hosts with intact pituitaries. We may conclude that DMBA is able to bring about follicular atresia and to render ovaries preneoplastic in the absence of pituitary stimulation.

Although hypophysectomy did not affect the ability of DMBA to render mouse ovaries "preneoplastic", it certainly prevented preneoplastic ovaries of intact DMBA-treated animals from developing into tumours when transplanted to hosts whose pituitaries had been removed. The sequence of events in the latter situation can also be followed.

The ovaries of 12 normal F_1 C57Bl × IF mice aged 6 to 7 months weighed 7·0 to 11·0 (mean 8·6) mg. They were pale pink and studded with bright pink, white or pale yellow spots which represented large follicles and corpora lutea in various stages of maturation. On section, such ovaries are seen to be composed mainly of corpora lutea with some maturing follicles at the periphery (Fig. 7). Atretic follicles are also present and there are small groups of cells containing accumulations of ceroid pigment (Marchant, 1959*a*).

The ovaries of mice of similar age which had just completed 3 months DMBA treatment weighed 2·0 to 6·5 (mean 3·8) mg. They were fairly smooth and yellowish. The previous experiment (Marchant, 1959*a*) showed, on sectioning such ovaries, an almost complete disappearance of oocytes and developing follicles and some degree of fusion of corpora lutea (Fig. 8). Atretic follicles were present and in 1 of 20 ovaries an early tumour nodule was found. Grafting of such ovaries orthotopically into mice with intact pituitaries has been shown to result in their development into ovarian tumours in a high proportion of cases within 15 months (Marchant, 1959*b*). But, in the present experiment 2b, grafting to hypophysectomised hosts resulted in their atrophy and degeneration (Fig. 9). Cysts were formed in some cases (Fig. 10) but no tumours occurred, although in the majority of cases there was eventually some evidence of regeneration of pituitary fragments. The process of tumour appearance and growth from preneoplastic ovaries was prevented by the absence of an intact pituitary in the host.

It is concluded that the development of ovarian tumours in mice after DMBA treatment may be divided into two distinct phases. The first phase is one of atrophy resulting from an increase in the rate of atresia of oocytes and follicles in the ovary. It is brought about by the action of DMBA and is quite independent of any action of the pituitary. The second phase of tumour development from such ovaries requires pituitary stimulation of some kind. In most DMBA-treated mice pituitary hyper-stimulation would eventually occur as a result of failure of ovarian hormones from the atrophic ovary.

SUMMARY

The effect of hypophysectomy on the induction of ovarian tumours in F_1 C57Bl × IF mice treated with 7,12-dimethylbenz(*a*)anthracene (DMBA) has been studied. The carcinogen proved toxic to hypophysectomised mice. It was possible to show that hypophysectomy did not prevent DMBA treatment from rendering ovaries preneoplastic: ovaries from such mice were able to develop into tumours when grafted orthotopically into hosts with intact pituitaries. On the other hand, preneoplastic ovaries grafted orthotopically from DMBA-treated to hypophysectomised hosts failed to develop into tumours.

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THE PRODUCTION OF MAMMARY TUMOURS IN RATS BY FEEDING WITH 3-METHYLCHOLANTHRENE

P. M. DANIEL AND MARJORIE M. L. PRICHARD

From the Department of Neuropathology, Institute of Psychiatry, Maudsley Hospital, London, S.E.5, and the Nuffield Institute for Medical Research, University of Oxford

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FOR some time we have been interested in the influence of the pituitary gland on growth, and have been studying the effects on growth produced by transection of the pituitary stalk. Since this operation causes massive infarction of the anterior lobe of the pituitary (Daniel and Prichard, 1957, 1958; Daniel, Prichard and Schurr, 1958) we have wondered whether pituitary stalk section might not be as effective as hypophysectomy in the treatment of those types of carcinoma for which the latter operation is at present used in human patients. We therefore wished to carry out some experiments on animals to determine the relative effects of the two operations on the behaviour of hormone-dependent tumours.

Some years ago, in an attempt to produce gastric tumours, Shay, Aegerter, Gruenstein and Komarov (1949) gave small does of methylcholanthrene daily by stomach tube to Wistar rats over a long period. Gastric tumours did not occur, but eventually mammary tumours developed in many of the rats, particularly the females, a finding confirmed in a later series of experiments (Shay, Harris and Gruenstein, 1952). This discovery was carried an important stage further by Huggins, Briziarelli and Sutton (1959), who gave 3-methylcholanthrene by stomach tube to Sprague-Dawley female rats, and found that under optimal conditions, including the right dosage and frequency of administration, mammary tumours could be obtained in all animals within 60 days. Since Huggins and his collaborators (1959) further observed that the majority of the tumours were hormone-dependent, we have used their method to provide material for our study.

In this paper we report the results which we have obtained in so far as the production of tumours is concerned.

METHODS

The carcinogen, 3-methylcholanthrene, was given by stomach tube to female albino rats of the Sprague-Dawley strain which had been bred in our laboratories. It was administered in a dose of 10 mg. three times a week (Monday, Wednesday, Friday) for a period of $7\frac{1}{2}$ weeks, (23 doses). The 3-methylcholanthrene was given in olive oil (sesame oil, as used by Huggins, being unobtainable at the time). The solution was made up by heating the olive oil with the added methylcholanthrene to a temperature of 140–160° C. for 30–60 min. We found that when the 3-methylcholanthrene was dissolved in a ratio of 10 mg. per ml. it remained in solution at body temperature, but precipitated out at room temperature, having to be reheated before administration. Accordingly, it was found more convenient to use a concentration of 10 mg. per 1·5 ml., at which strength it remained in

solution at room temperature. For a stomach tube we used a soft rubber urethral catheter, size 3 English gauge.

The experiments were carried out on 6 groups of rats, totalling at the beginning of the carcinogen-feeding 178 rats, and at the end (due to losses by infection or accident) 163 rats. In two groups of rats the age of the rats at the start of feeding was approximately 7–8 weeks and in the other four groups, the age ranged from $4\frac{1}{2}$ to $6\frac{1}{2}$ weeks. (The youngest rats, weighing about 85 g. at the start of feeding, were given only 7·5 mg. of 3-methylcholanthrene per dose until they had reached a body weight of 100 g.).

Throughout the experiments the rats were maintained on Blue Cross Diet 41B, with water *ad libitum*.

During the period of carcinogen-feeding and afterwards the rats were palpated at frequent intervals in order to determine the earliest development of tumours. The presence of a tumour was not recorded until a nodule of at least the size of a small pea was felt.

For histological examination specimens of the tumours were obtained either as biopsies (under ether anaesthesia) or at post-mortem in rats killed with chloroform. These specimens were fixed in formol alcohol (10 per cent formalin, 60 per cent alcohol). Blocks were embedded in paraffin and sections cut at 7μ. They were stained with haematoxylin and eosin and by Van Gieson's method.

RESULTS

As shown in Table I, mammary tumours developed in 149 rats out of the 163 animals which had been fed with 3-methylcholanthrene for $7\frac{1}{2}$ weeks. Thus, in these experiments, 91·4 per cent of the animals which had been given the carcinogen developed tumours.

TABLE I.—*Incidence of Mammary Tumours in* 163 *Rats Fed* 10 *mg. of 3-methylcholanthrene Thrice Weekly for* $7\frac{1}{2}$ *Weeks*

Group number of rats	Number of of rats fed carcinogen	Rats which developed tumours		Rats which did not develop tumours	
		Number	Time after end of carcinogen-feeding	Number	Period of survival after end of carcinogen-feeding
1	12	10	2–8 weeks	2	13, 13 weeks
2	35	33	3–19 weeks	2	4, 19 weeks
3	19	18	1·5–8 weeks	1	28 weeks
4	34	33	1·5–31 weeks	1	26 weeks
5	33	29	0–27 weeks*	4	21, 21, 29, 33 weeks
6	30	26	2–40 weeks	4	1, 9, 23, 42 weeks
Total number of rats	163	149		14	

* Two rats in this group had palpable tumours 2 days before the end of the period of carcinogen-feeding.

The earliest time at which a tumour was observed was 2 days before the end of the period of administration of the carcinogen. This, however, was unusual and, in general, it was only after the whole period of carcinogen-feeding had been completed that rats began to have palpable tumours. The number of rats develop-

ing tumours increased up to 6 weeks after the last dose of carcinogen had been given (Fig. 1). Thereafter the numbers fell. By the end of the 12th week no less than 130 (87·2 per cent) of the 149 rats had developed their tumours. In the remaining 19 rats tumours developed at various times up to 40 weeks after the last dose of carcinogen.

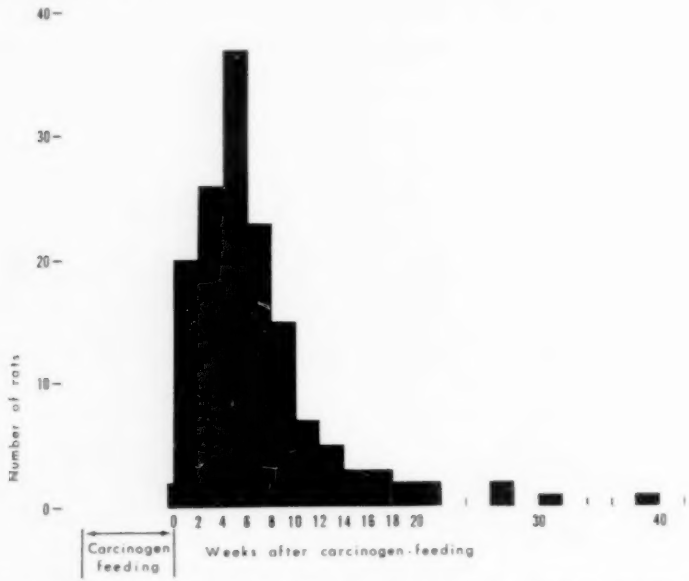


FIG. 1.—Histogram showing number of rats, in successive 2-week periods after carcinogen-feeding, presenting palpable tumours for the first time. Out of 163 rats fed with the carcinogen for 7½ weeks, 149 developed mammary tumours.

The 14 rats which are shown in Table I as rats which did not develop tumours were killed because of intercurrent infection (respiratory or middle ear) or for other reasons. Three of these were killed at 1, 4 and 9 weeks after the end of the period of carcinogen-feeding and therefore might well have developed tumours had they survived longer. Of the remaining 11 rats, which survived for from 13 to 42 weeks after the last dose of the carcinogen, it seems likely that few would at this late stage have developed tumours.

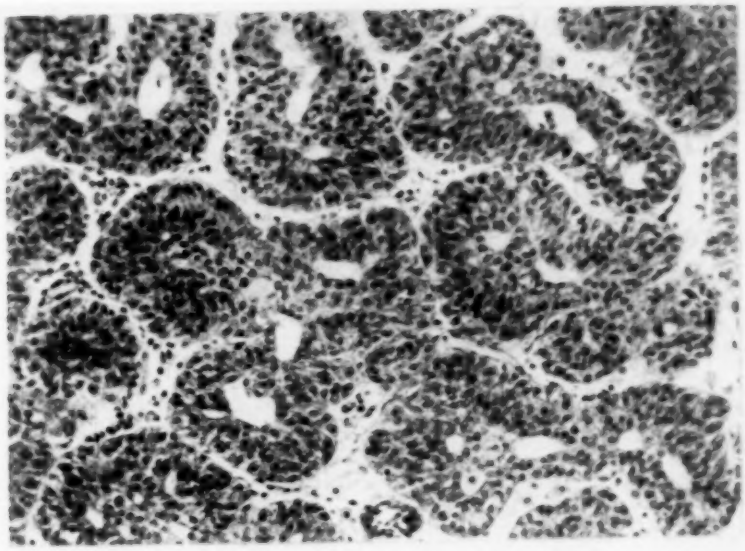
EXPLANATION OF PLATES

FIG. 2.—Typical appearance of mammary tumour in a rat which had been fed with 3-methylcholanthrene. H. and E. ×180.

FIG. 3.—In some tumours tracts of interstitial tissue separate groups of acini. H. and E. ×180.

FIG. 4.—Mammary tumour showing invasion of muscle. H. and E. ×180.

FIG. 5.—Numerous mitotic figures in mammary tumour. H. and E. ×440.



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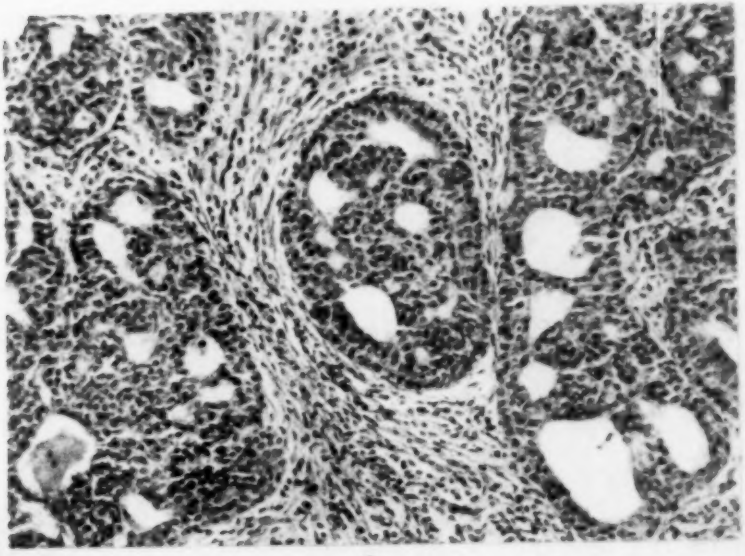

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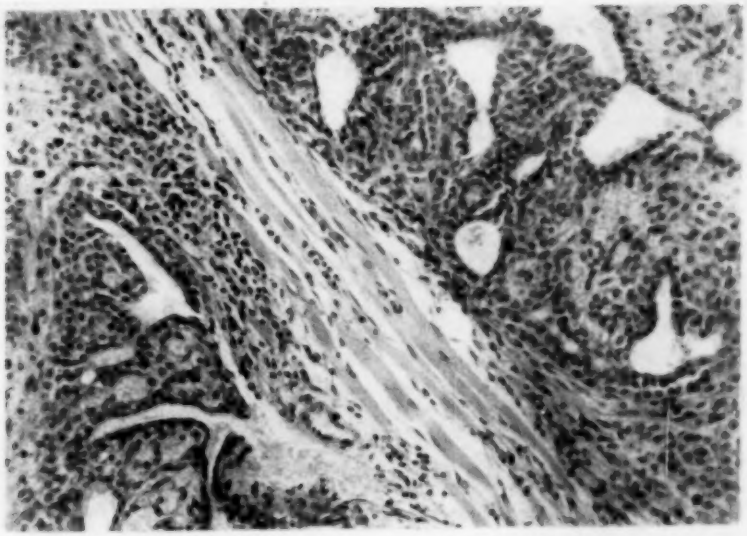

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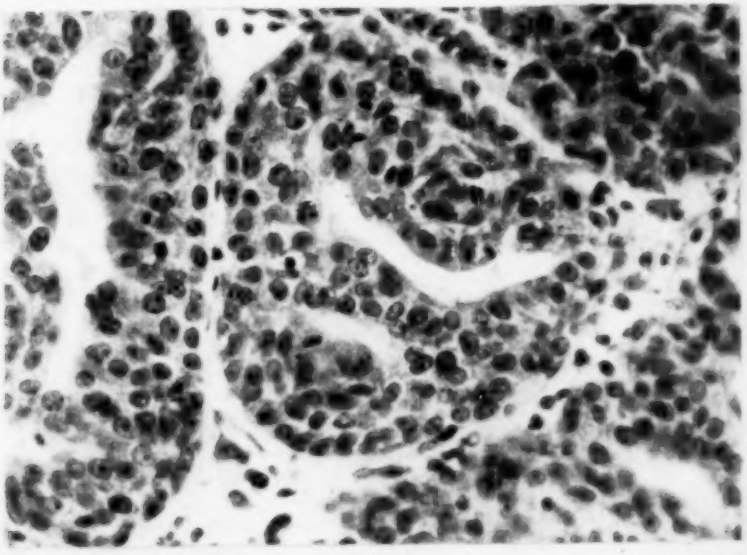

5

In several hundred female Sprague-Dawley rats we have only seen two spontaneous tumours and these were fibroadenomata in rats older than those of the present series.

All the tumours which developed in this series of rats occurred in the lower neck, pectoral, thoracic, abdominal or inguinal regions, i.e., roughly along the so-called " milk-line ". Some rats had single tumours but most had more than one and sometimes up to half a dozen or more.

Many of the tumours were rapidly growing, especially those which developed soonest after the carcinogen-feeding. Untreated these rapidly growing tumours would quickly attain a diameter of up to 4 or 5 cm. (up to 40 g. in weight) often with extensive cyst formation. These large tumours tended to ulcerate and when this happened the animal was killed. At no stage, did these tumours, however big or in however awkward a site, appear to distress or even inconvenience the rat. On palpation the great majority of tumours were firm. As they grew they became nodular, and they usually had flat bases (i.e., they were bun-shaped); their limits were clearly circumscribed.

Histologically, these tumours were characteristic adenomata, with malignant features. They were composed of large clumps of acini whose walls were made up of plump epithelial cells in which mitotic figures were often extremely numerous. The walls of the acini were frequently many cells thick (Fig. 5). In some tumours extensive tracts of very cellular interstitial tissue separated groups of acini (Fig. 3), but in other tumours little connective tissue was seen (Fig. 2). In the large tracts of interstitial tissue eosinophilic polymorphonuclear cells were very numerous and in some animals mast cells were a prominent feature, lying in between the acini of tumour cells. In advanced tumours necrosis and cyst formation were commonly seen, and in many instances mucoid degeneration was present. Invasion of muscle was not an uncommon finding (Fig. 4), but distant metastases were not found.

A very small percentage of tumours felt rather soft and flabby on palpation, and when cut, either at biopsy or at post-mortem, were found to be tough and stringy. These tumours proved to be fibro-adenomata; they all developed relatively late, i.e., several months after the end of the carcinogen-feeding, and the rats in which these fibro-adenomata were found had one or more other tumours of the purely adenomatous type.

DISCUSSION

While our results are in general agreement with those of Huggins *et al.* (1959), we have not been quite as successful as these workers in producing mammary tumours in rats by the administration of 3-methylcholanthrene. Huggins and his collaborators found that by feeding 3-methylcholanthrene to rats in a dosage of 10 mg., given by stomach tube at a frequency of either 1, 2, 3, 4, 5 or 6 days a week for 50 days, they obtained tumours in every rat. Moreover, the tumours in their rats developed more quickly than those in our series, many appearing within 30 days of *starting* the feeding with the carcinogen. In our experiments, with a thrice-weekly administration of 10 mg. 3-methylcholanthrene for 51 days, approximately 90 per cent of the animals developed tumours, but virtually all of the tumours appeared only after the end of the period of carcinogen-feeding, the peak period being between 2 and 8 weeks after the end of feeding (Fig. 1).

We do not know why our results are not quite as good as those of Huggins *et al.* (1959). We used the same strain of rats, and our dosage and frequency of administration fell within the scheme recommended by them. There were, however, some minor points of difference in our technique. Firstly we used olive oil instead of sesame oil as the vehicle for the carcinogen, and secondly, many of our rats were younger at the time when the carcinogen-feeding was started. However, we found that there was no obvious difference in the development of tumours between the older and the younger rats. We agree with Huggins *et al.* (1959) that the carcinogen given in this dosage does not appear to have any toxic effect on the rats, but we have the impression that our carcinogen-fed animals were more susceptible to respiratory infections than normal rats of the same strain. Possibly this was related to the repeated intubation.

However, even though our results are not quite so satisfactory as those of Huggins and his colleagues, they do show that the oral administration of 3-methylcholanthrene produces mammary tumours in rats quickly and in a high percentage of animals. Huggins *et al.* (1959) found that the majority of tumours in their rats were hormone-dependent, an observation supported by the work of Dao and Sunderland (1959). A study which we have been making of the effects of hypophysectomy, or of cutting the pituitary stalk, on the behaviour of the tumours indicates that in our experiments also most of the tumours produced by this method are hormone-dependent.

SUMMARY

Oral administration of 3-methylcholanthrene given in a dosage of 10 mg. 3 times a week for $7\frac{1}{2}$ weeks to 163 female rats of the Sprague-Dawley strain produced mammary tumours in 149 (91·4 per cent) of the animals. Most of the tumours developed within 12 weeks of the end of the period of the carcinogen feeding.

We are greatly indebted to Dr. Charles Huggins for his encouragement and for sending us some of his Sprague-Dawley rats with which to start a colony. Our thanks are also due to Mr. Edward Bernard and Miss J. Booty who gave much help with the rats, and to Miss J. Smith and Mr. R. Jeacock for cutting the sections. Dr. O. E. Pratt has also helped us in a number of ways. The work was supported by a grant from the British Empire Cancer Campaign for which we are very grateful.

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THE EFFECT OF THE WALKER 256 CARCINOMA ON HEPATIC Δ^4-STEROID HYDROGENASE ACTIVITY

G. A. J. GOODLAD AND C. M. CLARK

From the Department of Physiology and Biochemistry, St. Salvator's College, University of St. Andrews, Fife

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THE reduction of Ring A of adrenal steroids by the Δ^4-steroid hydrogenases of liver has been shown in man (Peterson, Wyngaarden, Guerra, Brodie and Bunim, 1958) and in the rat (Glenister and Yates, 1961) to be the rate-controlling step in the inactivation of corticosteroids. Urquhart, Yates and Herbst (1959) have further shown that there is a close correlation between adrenal size and the level of this enzyme system in the liver. Thus under experimental conditions where a rise in the activity of this enzyme was induced, for example by treatment with thyroxine (Yates, Urquhart and Herbst, 1958) or castration of male rats (Yates, Herbst and Urquhart, 1958), there was an increase in adrenal weight; conditions which produced a reduction in enzymic activity, such as partial hepatectomy (Urquhart *et al.*, 1959), caused adrenal atrophy. Administration of corticotrophin or cortical hormones, on the other hand, did not increase the inactivating capacity of the liver (Urquhart *et al.*, 1959). These authors therefore concluded that it is the level of hepatic Δ^4-steroid hydrogenase activity which determines adrenal size rather than the reverse.

Since adrenal hypertrophy is a common feature of tumour-bearing animals (Begg, 1958) the present study was undertaken to determine whether the presence of a growing tumour affected the rate of hepatic reduction of Ring A of adrenal steroids.

EXPERIMENTAL METHODS

Animals

Virgin female albino rats were fasted overnight and those weighing 140–160 g. were selected and housed in individual cages.

Treatment of animals

The animals were anaesthetised with ether and the experimental group injected intramuscularly into the right thigh with 1 ml. portions of a suspension of Walker 256 carcinoma, prepared as described by Talalay, Takano and Huggins (1952). The control group received an intramuscular injection of 1 ml. 0·9 per cent saline.

Throughout the experimental period both groups of rats were fed a diet adequate in calories and protein content as described previously (Clark and Goodlad, 1960).

The rats were killed at various time intervals after injection by stunning and exsanguination. The liver, adrenal glands and tumour were rapidly removed and weighed.

Determination of hepatic Δ^4-steroid hydrogenase activity

Liver slices, prepared using the tissue slicer described by McIlwain and Buddle (1953), were assayed for Δ^4-steroid hydrogenase activity essentially as described by Urquhart *et al.* (1959) save that the amount of slice assayed was 50 mg. and the amount of corticosterone added to each assay was 0·55 μmoles. Under these conditions it was found that the rate of reduction of corticosterone was proportional to the time of incubation during the first 30 minutes and the enzyme activity proportional to the weight of slice between 50–100 mg.

The reaction was stopped by the addition of 20 ml. redistilled methylene chloride and the steroid quantitatively extracted by shaking. The amount of corticosterone reduced was measured as described by Tomkins (1957).

RESULTS

The assay of hepatic Δ^4-steroid hydrogenase activity was carried out using corticosterone as substrate since this is the principle steroid secreted by the rat adrenal (Bush, 1953).

The size of the Walker 256 carcinoma at the various times of killing are shown in Table I. At 30 hours it was impossible to distinguish any tumour tissue.

TABLE I.—*Tumour Weight at Various Time Intervals After Tumour Inoculation*

Results are the means of 6 experiments ± S.E.

	Time after injection			
	30 hours	3 days	5 days	7 days
Tumour weight (g.)	—	0·1 ± 0·08	2·9 ± 0·5	9·7 ± 0·9

This was also the case on the third day save for two of the six animals which had tumours of 0·2 g. and 0·4 g. respectively. By the fifth day the growth of the tumour was well established.

TABLE II.—*The Effect of the Walker 256 Carcinoma on the Concentration of Hepatic Δ^4-Steroid Hydrogenase Activity*

Results are expressed as the means ± S.E. Figures in parentheses refer to the number of animals in each group

μmoles corticosterone reduced/15 min./g. liver slice

	Time after injection			
	30 hours	3 days	5 days	7 days
Control group	2·28 ± 0·30 (4)	2·05 ± 0·15 (5)	1·87 ± 0·27 (6)	1·81 ± 0·09 (6)
Tumour-bearing group	2·54 ± 0·15 (4)	2·30 ± 0·12 (6)	2·40 ± 0·18 (6)	1·96 ± 0·19 (6)
P*	>0·40	>0·20	>0·10	>0·40

* Statistical analysis in this and subsequent tables carried out by Student's "t" test.

Table II shows the concentration of hepatic Δ^4-steroid hydrogenase activity (μmoles corticosterone reduced/15 min./g. liver slice) at several time intervals after injection. There was no significant difference between the control and

tumour-bearing groups at any of the time intervals studied. There was also no significant variation within either group with time.

The effect of tumour growth on the total hepatic Δ^4-steroid hydrogenase activity (μmoles corticosterone reduced/15 min./liver/100 g. initial body weight) is shown in Table III. There was a significant increase in the total activity as

TABLE III.—*The Effect of the Walker 256 Carcinoma on the Total Hepatic Δ^4-Steroid Hydrogenase Activity*

Results are expressed as the means ± S.E. Figures in parentheses refer to the number of animals in each group

μmoles corticosterone reduced/15 min./liver/100 g. initial body weight

	Time after injection			
	30 hours	3 days	5 days	7 days
Control group	7·01 ± 1·10 (4)	5·96 ± 0·50 (5)	5·86 ± 0·96 (6)	5·44 ± 0·23 (6)
Tumour-bearing group	10·30 ± 0·67 (4)	8·43 ± 0·68 (6)	10·22 ± 1·37 (6)	9·04 ± 1·34 (6)
P	<0·05	<0·02	<0·05	<0·05

early as 30 hours after injection of tumour and this increase was maintained throughout the period of tumour growth studied. This early rise in enzyme activity was associated with enlargement of the livers of these animals (Table IV).

TABLE IV.—*The Effect of the Walker 256 Carcinoma on Liver Weight*

Results are expressed as the means ± S.E. Figures in parentheses refer to the number of animals in each group

g. liver/100 g. initial body weight

	Time after injection			
	30 hours	3 days	5 days	7 days
Control group	3·04 ± 0·11 (4)	2·90 ± 0·17 (5)	3·09 ± 0·07 (6)	3·02 ± 0·10 (6)
Tumour-bearing group	4·06 ± 0·08 (4)	3·64 ± 0·13 (6)	4·22 ± 0·29 (6)	4·50 ± 0·28 (6)
P	<0·01	<0·01	<0·01	<0·01

The results in Table V indicate that this increased activity of the Δ^4-steroid hydrogenase was accompanied by adrenal hypertrophy. The adrenals showed a significant increase in weight at 30 hours and this was maintained throughout the 7-day period.

DISCUSSION

The total Δ^4-steroid hydrogenase activity is increased in the livers of rats bearing the Walker 256 carcinoma. This increase is observed as early as 30 hours after tumour inoculation and is accompanied by an increase in adrenal size.

TABLE V.—*The Effect of the Walker 256 Carcinoma on Adrenal Weight*

Results are expressed as the means ± S.E. Figures in parentheses refer to the number of animals in each group

mg. adrenal/100 g. initial body weight

	Time after injection			
	30 hours	3 days	5 days	7 days
Control group	29 ± 1 (4)	30 ± 1 (5)	31 ± 2 (6)	33 ± 1 (6)
Tumour-bearing group	40 ± 3 (4)	39 ± 2 (6)	42 ± 1 (6)	45 ± 4 (6)
P	<0·05	<0·01	<0·01	<0·02

Ball and Samuels (1938) failed to observe adrenal hypertrophy in hypophysectomised rats bearing the Walker 256 carcinoma, a finding which indicates that the increase in adrenal size is due to an increased production of corticotrophin (Begg, 1958).

The level of circulating corticosteroids influences the control of the secretion of corticotrophin by a negative feedback mechanism (Sayers and Sayers, 1947) and Urquhart *et al.* (1959) have demonstrated that hepatic inactivation of adrenal corticoids by Ring A reduction can be a principal determinant in this control.

Our experimental conditions do not exclude the possibility that the adrenal hypertrophy may occur through a direct action on the hypothalamus-pituitary system and that the observed increase in hepatic Δ^4-steroid hydrogenase activity is merely an adaptation to an increased availability of substrate. However it must be pointed out that direct attempts to simulate wide variations in adrenal-cortical function did not cause parallel changes in the capacity of the liver to inactivate corticosteroids (Urquhart *et al.*, 1959). We would suggest therefore that the marked increase in hepatic Δ^4-steroid hydrogenase activity observed during the growth of the Walker 256 carcinoma is due to a direct action on this enzyme system and that this effect plays a significant role in the production of adrenal hypertrophy in the tumour-bearing rat.

The mechanism whereby the Δ^4-steroid hydrogenase activity is raised cannot be ascertained from the present work. However the fact that we have observed these effects so soon after implantation of the tumour suggests that the initial changes in hepatic enzyme activity might be caused by some factor present in the tumour inoculum.

SUMMARY

1. The effect of the Walker 256 carcinoma on hepatic Δ^4-steroid hydrogenase activity was studied in rats during the first 7 days of tumour growth.

2. The total hepatic Δ^4-steroid hydrogenase activity was increased markedly as early as 30 hours after implantation of the tumour and elevated levels were observed throughout the period studied.

3. This increase in hepatic enzyme activity was accompanied by an increase in adrenal weight.

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THE CARCINOGENIC ACTION OF COMPLEX IRON PREPARATIONS

P. M. LUNDIN

From the Department of Pathology, University of Gothenburg, Sweden

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FROM experimental and clinical observations it has long been known that certain metals—such as nickel, arsenic and beryllium—may exhibit a carcinogenic action. However, as a carcinogenic substance iron has attracted comparatively scant attention. Nevertheless Kennaway and Kennaway (1936) demonstrated that iron and steel grinders had 2·5 times as high an incidence of lung cancer as the population at large. Faulds (1954) found that lung cancer had a prevalence of 8·85 per cent (17 of 192) amongst iron mine workers. The latter figure may be compared with an incidence of lung cancer in the total population of 1·85 per cent. In spite of these previous findings, much comment ensued following Richmond's (1959) report that sarcoma was induced in albino rats after intra-muscular injections of iron in the form of an iron-dextran complex ("Imferon"). His results have been discussed at length in the light of their theoretical significance but also and mainly on account of their repercussions on human medicine.

The tumours became apparent a fairly long time (8–16 months) after repeated injections of large iron-dextran doses.

Whilst experiments on rats and mice (Haddow and Horning, 1960; Golberg, Martin and Smith, 1960) have, experiments on rabbits (Haddow and Horning 1960) and dogs (Golberg, Martin and Smith, 1960) have not confirmed Richmond's results. In a more comprehensive series of trials on mice, Haddow and Horning (1960) have studied the carcinogenic action of, among other things, various metal complexes. So far, however, most of the tested substances have shown no carcinogenic action, although various aluminium-dextran and copper-hydroxy quinoline complexes have induced some sarcomas.

Richmond (1960) suggested that a relationship exists between the local iron-dextran dose and the incidence of tumours. Golberg, Martin and Smith's (1960) observations also suggest that this is so. Richmond (1960) considered it obvious that iron was the carcinogenic component of the complex. And, despite diligent efforts, several workers have failed to induce tumours with low molecular weight dextran alone. The presence of dextran could be a prerequisite for the penetration of iron into the cells (phagocytosis). Whereas Richmond was unable to induce sarcomas on mice after iron-saccharate complex, Haddow and Horning did observe a few sarcomas in mice following injections of that substance.

The present investigation had for its aim to compare the carcinogenic actions in the rat of intramuscular injections of three iron complexes of different types. Apart from dissimilarities in chemical composition, these iron complexes deviated with respect to molecular size. Partly owing to the latter deviation, the three substances differed in their rates of absorption from the site of the injection.

Iron complexes

" Ferrigen " syn. " Astrafer " (Astra): A high molecular weight iron-carbohydrate complex.* The average sedimentation constant has been determined to be 47·5 ± 2·5 Svedberg uni's. Assuming that the molecules are spherical with a density of 3·4 this corresponds to an average molecular weight of 230,000 (Eriksson).

" Imferon "(Pharmacia, Benger): A complex of low molecular weight dextran and iron.* Preliminary experiments indicate that the average sedimentation constant of this preparation is 10 to 20 per cent smaller than that of " Ferrigen " which with the assumptions mentioned gives an average molecular weight of 180,000 (Eriksson).

" Jectofer " (Astra): An iron-sorbitol-citric acid comples, stabilized with dextrin.* The upper limit of the sedimentation constant has been found not to exceed 8 to 9 Svedberg units. The average molecular weight has been estimated to be less than 5,000 (Eriksson).

All the preparations have a concentration of 50 mg. iron per ml. and were used without dilution.

MATERIALS AND METHODS

Albino rats of the Sprague-Dawley strain (Anticimex, Stockholm) were used Initially the rats were about 40 days old and weighed between 60 and 80 g. They were fed commercial rat bread reinforced with supplementary vitamins and salts. The rats were injected (into the muscles of the right thigh) with rising doses twice weekly for 4 months in accordance with the following schedule, in principle after Richmond (1960).

	Body weight (g.)				Mean total dose per rat (mg.)
	<100	100–150	150–200	>200	
Large dose, ml. . .	0·1	0·2	0·3	0·4	510
Small dose, ml. . .	0·05	0·1	0·15	0·2	255

Experimental groups

I. Untreated control rats
II. Rats injected with large Iron-dextrin (Ferrigen) dose
III. Rats injected with small Iron-dextrin (Ferrigen) dose
IV. Rats injected with large Iron-dextran (Imferon) dose
V. Rats injected with small Iron-dextran (Imferon) dose
VI. Rats injected with small Iron-sorbitol (Jectofer) dose
VII. Rats injected with " Ferrigen " carbohydrate (dextrin) component in amount corresponding to large dose.

Owing to the comparatively high toxicity of " Jectofer ", it could not be administered in the high dose. Initially each group included some 40 rats, with approximately equal numbers of males and females.

When the experiment had been in progress for a month, altogether 4 or 6 rats from each group were killed at intervals of 3 or 4 weeks to facilitate observation of any early lesions. At the autopsy, the muscles, all parenchymatous, lymphoid

*According to the manufacturer's specification.

and endocrine organs, and bone marrow were saved. The specimens were fixed in neutral formalin, embedded in paraffin in the usual manner, and stained in accordance with van Gieson's technique and a modified Prussian blue procedure due to Beutler, Robson and Butterwieser (1958).

In some of the rats the iron contents of muscles, liver, spleen and lymph nodes were determined with the aid of the *o*-phenanthrolin method after the dried tissues had been ashed with sulphuric acid.

RESULTS

After some weeks of injections, the injected thigh became swollen and seemed rather tender in all groups receiving iron. In due course the swelling became rather marked, particularly in those groups with " Imferon " and " Ferrigen " in

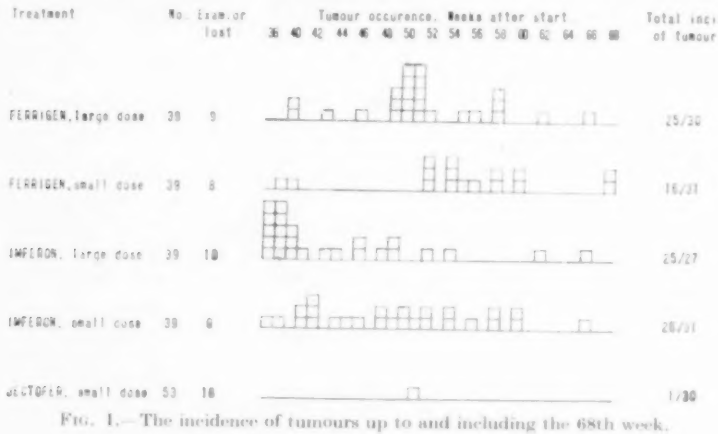

Treatment
No. Exam.or lost
Tumour occurence. Weeks after start
36 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68
Total incid. of tumour
FERRIGEN, large dose 39 9 25/30
FERRIGEN, small dose 39 8 16/31
IMFERON, large dose 39 10 25/27
IMFERON, small dose 39 6 26/31
JECTOFER, small dose 53 16 1/30

FIG. 1.—The incidence of tumours up to and including the 68th week.

large doses. On the other hand, the swelling was much smaller in the "Jectofer " group. After a few months of treatment, the skin of some of the rats in the " Ferrigen " groups became necrotized and ulcerated, but such lesions would heal spontaneously in a week or two.

When the injections were discontinued at the end of the fourth experimental month, the local swelling diminished. Although it vanished almost completely in the rats from the " Jectofer " group, a distinct induration persisted in the " Imferon " groups as well as in the " Ferrigen " groups.

Tumour development.—The incidence of tumours up to and including the 68th week is shown in Table I and Fig. 1. The " Imferon " groups exhibited the highest incidence of tumours, the time of onset of tumours in the small dose group being somewhat later than in the large dose group. But at the 68th week the two groups showed the same total incidence. The males surviving from the group receiving the large " Ferrigen " dose showed a similar high incidence of tumour development However, the group treated with the small " Ferrigen " dose displayed a much lower frequency of tumours which differed significantly from the group receiving small " Imferon " doses ($P < 0{\cdot}01$). Furthermore, after small " Ferrigen "

TABLE I.—*Incidence of Tumours up to and Including the 68th Week*

	Number of rats from the start	Rats lost during the trial	Examined: With tumour	Examined: Without tumour	Surviving: With tumour	Surviving: Without tumour	Time occurrence of tumours (Weeks after beginning of the trial) 38	40	42	44	46	48	50	52	54	56	58	60	62	64	66	68	Incidence of tumour
Normal, untreated																							
♂	20	1	—	7	—	12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
♀	19	—	—	8	—	11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ferrigen, large dose																							
♂	20	2	13	4	1	—	—	—	—	—	1	3	8	—	1	1	—	—	—	—	—	—	14/15
♀	19	—	10	8	1	—	—	2	1	—	—	—	3	—	—	—	3	—	1	—	1	—	11/15
Ferrigen, small dose																							
♂	20	1	3	5	5	6	—	—	—	—	—	—	—	1	1	1	1	2	—	—	—	2	8/16
♀	19	2	5	4	3	5	1	1	—	—	—	—	—	2	3	—	1	—	—	—	—	—	8/15
Imferon, large dose																							
♂	20	2	12	5	1	—	7	1	1	1	—	2	—	—	—	—	—	—	1	—	—	—	13/14
♀	19	2	10	5	2	—	6	—	—	—	2	1	—	1	1	—	—	—	—	—	1	—	12/13
Imferon, small dose																							
♂	20	1	12	5	1	1	—	2	2	2	1	1	3	—	—	—	—	2	—	—	—	—	13/15
♀	19	—	11	5	2	1	2	—	1	—	—	2	—	2	2	1	2	—	—	—	1	—	13/16
Jectofer, small dose																							
♂	28	5	1	8	—	14	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	1/19
♀	25	10	—	9	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0/11
Carbohydrate																							
♂	20	5	—	7	—	8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
♀	20	3	—	8	—	9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

doses the tumours tended to set in later than after large " Ferrigen " doses ($P < 0{\cdot}01$).

Moreover, in both " Ferrigen " groups the tumours showed a tendency to later onset than in either of the " Imferon " groups. In this respect a significant difference exists between the combined " Imferon " groups and the combined " Ferrigen " groups ($P < 0{\cdot}001$).

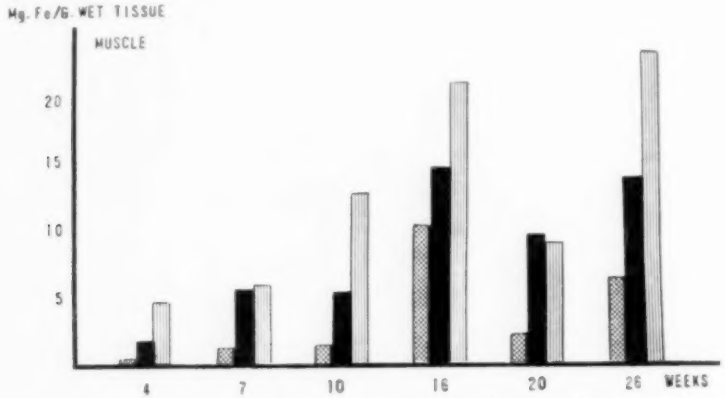


FIG. 2.—Iron concentrations locally in muscular tissue varying lengths of time after commencement of injections of "small doses " of iron complexes. Mg. per g. wet tissue.

Jectofer. Imferon. Ferrigen.

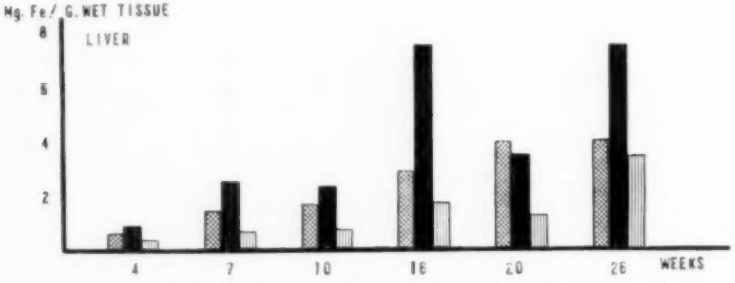


FIG. 3.—Iron concentrations in liver varying lengths of time after commencement of injections of " small doses " of iron complexes. Mg. per g. wet tissue.

Jectofer. Imferon. Ferrigen.

Among the rats in the " Jectofer" group a single tumour at injection site was encountered in the 51st week (see below). No tumours were observed among the controls or in the carbohydrate group.

The results of the iron determinations are shown graphically in Fig. 2 to 5. The rate of iron absorption from the site of the injection varied greatly among the three iron complexes. " Ferrigen " exhibited a distinctly higher concentration at the site of the injections after 4 to 6 months. Neither " Imferon " nor " Ferrigen "

seemed to yield local iron concentrations that tended to decline after the cessation of injections, although in this respect definite conclusions are not justified on the basis of the available data.

The iron concentrations of liver and spleen were much lower in the " Ferrigen " groups than in the " Imferon " groups, where these levels were of the same order as in the " Jectofer " group. The fact that, despite their superior absorption of

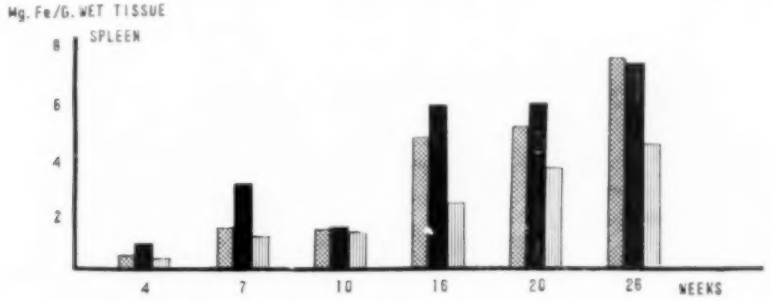


FIG. 4.—Iron concentrations in spleen varying lengths of time after commencement of injections of " small doses " of iron complexes. Mg. per g. wet tissue.

Jectofer. Imferon. Ferrigen.

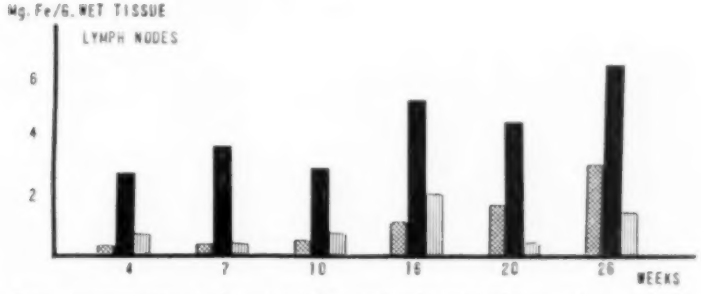


FIG. 5.—Iron concentrations in lymph nodes varying lengths of time after commencement of injections of " small doses " of iron complexes. Mg. per g. wet tissue.

Jectofer Imferon Ferrigen

iron from the site of the injection, the latter rats showed no higher iron levels in liver and spleen than the rats in the " Imferon " groups might be because the low molecular weight had enabled some of the " Jectofer " iron to be excreted via the kidneys.

Histological observations

The early lesions were studied in 4 to 6 rats from each group which were killed between 1 and 7 months after the commencement of the experiment. It was noted that although the appearance of the histological reaction was basically the same in all groups treated with iron, the rats in the " Jectofer " group deviated distinctly

from those in the other groups. As early as after the 1st month of injections the rats in the " Imferon " and " Ferrigen " treated groups displayed a profuse histiocytic reaction with accumulations interstitially in the muscles and, above all, in foci in the surrounding adipose tissue of numerous macrophages charged with phagocytized iron in abundance. This histiocytic reaction increased steadily up to the 4th month, and at the same time fibrosis appeared interstitially and in the perimuscular tissue. In several rats from the " Ferrigen " groups rather extensive necrotic areas with abscess formation were encountered.

In slides stained with Prussian blue the iron was seen chiefly as large and small granules in macrophages but also as a distinctly blue staining of collagen fibres and vascular walls. However, the muscle fibres contained merely minute, interfibrillar iron granules.

Between 4 and 6 months after the commencement of the experiment the histiocytic reaction had become still more pronounced, and perhaps somewhat more intense among rats from the " Ferrigen " treated groups than among those receiving " Imferon ". Progressive fibrosis was observed both interstitially and in the perimuscular adipose tissue. In some of the rats there appeared particularly in the connective tissue a measure of cellular polymorphism with some large nuclei of irregular shape and occasional mitoses (Fig. 6 and 7).

The majority of the histiocytes had regular, small nuclei and abundant cytoplasm, but here and there patches of these cells would also exhibit some degree of nuclear polymorphism.

Consistently and from the very beginning of the experiment those rats which had been injected with " Jectofer " displayed a distinctly less intense histiocytic reaction than those from the other groups. On the other hand, at an early stage the amount of iron deposited in collagen tissue and vascular walls seemed greater than in any of the other 4 groups receiving iron. The fibrosis, particularly that occurring perimuscularly, appeared to be equally marked in the " Jectofer " rats and in the rats from the other groups.

Tumour morphology.—The fully developed tumours were round, firm bodies which over a period of 2 to 5 weeks grew to the size of an orange and often ulcerated the skin. On being transected they were found fairly well circumscribed from the surrounding muscles and adipose tissue. Centrally the larger ones invariably exhibited necrotic foci with cystic degeneration. Large retroperitoneal metastases were observed in two rats and pulmonary and pericardiac metastases were encountered in one rat. One of the metastatic tumours had been " Imferon " induced, the two others were " Ferrigen " induced.

EXPLANATION OF PLATE

FIG. 6.—Accumulations of large, pigment-containing macrophages in perimuscular adipose tissue, 6 months after commencement of " Ferrigen " injections.

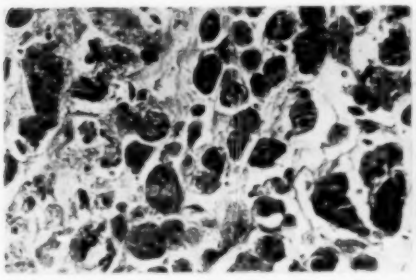
FIG. 7.—Intramuscular fibrosis and moderate nuclear polymorphism 8 months after commencement of " Ferrigen" injections.

FIG. 8.—Spindle cell sarcoma after " large dose " of " Imferon ".

FIG. 9.—Highly polymorphocellular sarcoma with " histiocytic " polynuclear giant cells induced by " large dose " of " Ferrigen ".

FIG. 10.—Muscular tissue infiltrated by predominantly spindle cell tumour tissue with abundant mitoses, induced by " large dose " of " Imferon ".

FIG. 11.—Moderately cell-rich fibroma induced by " Jectofer ".



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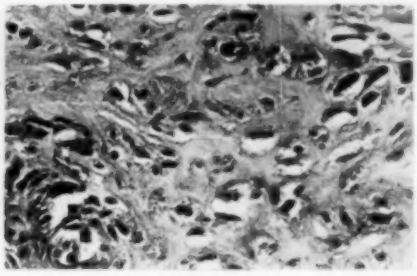

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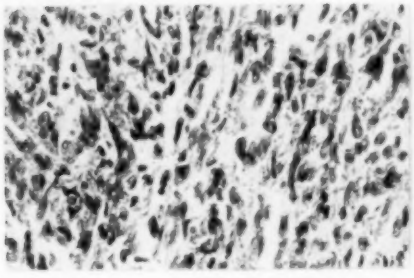

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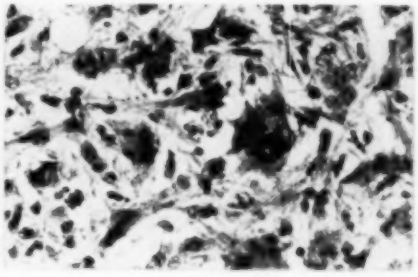

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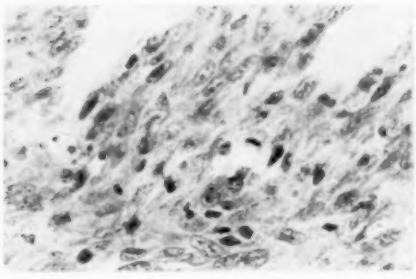

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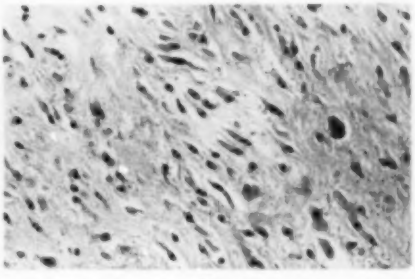

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At microscopic examination the tumours exhibited varying appearances. For the most part the picture was that of a low-differentiated tissue with an abundance of spindle cells, negligible collagen formation and fairly large numbers of mitoses (Fig. 8). Marginally the tumours infiltrated muscles and fat (Fig. 10). Occasional tumours were less rich in cells and distinctly collagen forming. Small areas with a definitely more pronounced cellular polymorphism could be observed in the majority of tumours. Some tumours exhibited a highly grotesque cellular polymorphism with large, often polynuclear cells containing giant nuclei and a profusely granulated cytoplasm, and large atypical mitoses (Fig. 9).

In the spindle celled tumours iron occurred predominantly in histiocytes having small nuclei and abundant cytoplasm which were interspersed among the tumour cells. Only minuscule quantities of iron were to be seen in the tumour cells themselves. Nevertheless, those tumours displaying the most accentuated cellular polymorphism invariably exhibited the presence of iron pigment also in these grotesquely polymorphic tumour cells.

The " Jectofer " induced tumour presented a deviating picture. Consistently very poor in cells, it exhibited small regular spindle cells without mitoses and lively collagen formation. Hence it distinctly differed from the " Ferrigen " and " Imferon " induced tumours and, on a purely histological basis, it should be classified more correctly as a fibroma (Fig. 11).

DISCUSSION

These results verify previous workers' observations regarding the carcinogenic action of intramuscularly administered iron-dextran. Moreover, they indicate that an iron-dextrin complex exerts a similar action in principle. Richmond's (1959, 1960) and Haddow's (1959) assumptions that the carcinogenic action is bound to the iron are thus borne out. Just as in their experiments, our trials have failed to disclose any carcinogenic action of the isolated carbohydrate component (dextrin).

The correlation of tumour incidence to injected dose was not so obvious in the present investigation. Yet a statistically significant difference pointing in that direction was present between the groups treated with large and small " Ferrigen " doses. In addition there was a tendency to later tumour onset in the groups receiving small " Ferrigen " and " Imferon " doses than in the corresponding high-dosage groups.

When the incidence of tumours is related to the iron concentration at the site of the injection, it appears that the number of tumours was definitely lower in the small-dose " Ferrigen " group than in the small-dose " Imferon " group, notwithstanding the fact that the local iron concentration of the muscles was higher in the former group. In addition, the rats from the " Ferrigen " groups displayed a later tumour onset than those from groups with corresponding dosages of " Imferon ".

The local iron concentration in the muscles of the " Jectofer " treated rats was clearly lower than in the rats treated with either high or low doses of " Ferrigen " and " Imferon ". Herein may lie the reason for the absence of sarcomas in the former group. Another possible prerequisite for the nondevelopment of sarcomas might be the considerably less marked histiocytic reaction which, at least to some extent, would seem to be a consequence of the low molecular weight of " Jectofer ".

Needless to say, the development of a fibroma-like tumour in this group must have been brought about by the injections.

Histogenesis.—In Richmond's opinion the tumours were most nearly classifiable as histiocytomas and he apparently believed them to arise from those histiocytes charged with iron pigment which proliferate in the muscle septa and perimuscular connective tissue. He also demonstrated that the polymorphic giant cells encountered in some of these tumours are susceptible to silver impregnation by Marshall's modification of Weil-Davenport's method. This may indicate that such cells possess histiocytic characteristics. Curiously enough, however, in the majority of tumours iron pigment is rather sparse in the tumour tissue itself, and by far the greater part of any pigment present is found in morphologically typical macrophages lacking signs of cellular polymorphism. On the other hand, large amounts of iron pigment are often encountered in the polymorphic giant cells.

In my experience, however, any cellular polymorphism seen in the " pre-malignant " phase can be observed most distinctly in the fibrosis which is particularly marked perimuscularly. Fibroblast-like, often polynuclear cellular elements with irregular large nuclei and also some mitoses were encountered in such regions some 6 to 9 months after the commencement of the experiment. The histiocytic elements seemed to exhibit a less conspicuous cellular polymorphism which, however, was more difficult to judge owing to the abundance of pigment present in these cells.

Accordingly the most prevalent tumour tissue component with densely packed spindle cells should perhaps be interpreted as fibrosarcoma. Everything suggests, however, that the designation histiocytic sarcoma is deserved by the highly polymorphic tumours with giant cells which often contain iron pigment and, according to Richmond, are positive to Marshall's silver impregnation.

The collagen fibrils in the muscle septa and vascular walls at an early stage consistently showed a uniformly intense, positive iron reaction. Consequently the carcinogenic action of iron can be exerted not only against the histiocytic cells but no doubt also against the fibroblasts.

Mode of action of iron.—Since the carcinogenic action of iron can be induced with the iron bound in different complexes, the iron component seems responsible for this action. Conceivably the other component is essential merely as a mediator facilitating the deposition of enough iron in the tissues and enabling it to be retained and slowly absorbed so that a high and constant iron concentration is maintained. Richmond expressed the view that the dextran component made it easier for the iron to enter the cell. The histiocytic reaction is surely dependent upon molecular size, and therefore upon molecular weight, and is unlikely to occur below a given minimum molecular size. In conformity with this we have found that " Jectofer "—whose molecular weight is much smaller than that of either " Ferrigen " or " Imferon "—induces a distinctly less marked histiocytic reaction by virtue of its smaller molecules than either of the other iron complexes tested.

In view of the high incidence of tumours in the muscles, it is a remarkable thing that tumours had not developed in the liver or spleen before the experiment was discontinued. The concentration of iron, especially in the " Imferon " group, was of the same order in the liver as in the muscles. Notably, however, the iron content of the liver was histologically found mainly in groups of very large macrophages the majority of which were located periportally, there being no appreciable

amounts of iron deposited in the collagen, stroma or vascular walls. Nor did we observe any proliferation of hepatic connective tissue, thus verifying the reports of Golberg *et al.* (1960) Yet one would expect the chances for the development of histiocytic tumours in the liver to be as great as at the site of the injection. Conversely, if most of the tumours are interpreted as being of fibrosarcomatous type, then the absence of fibrosis in the liver can well be reconciled with the absence of carcinogenic activity there. Haddow and Horning (1960) observed occasional hepatomas but reported no sarcomas or histiocytomas in the liver.

The mechanism of the carcinogenic action exerted by iron is of course just as obscure as that exerted by other carcinogenic substances. Richmond and Haddow discussed a variety of conceivable points of interference with cellular function, such as disturbed nucleic acid, vitamin E and haemoglobin metabolism or upsets in various enzymatic systems. Hueper (1957) postulated that the formation of macromolecular protein carcinogens might be a common denominator for the action of different types of carcinogenic substances. It is remarkable that Hueper also got tumours (in most cases malignant lymphomas) after injection in mice of high molecular weight dextran of different degree of branching of the molecule (Hueper, 1959).

SUMMARY

The carcinogenic actions of three different iron complexes intramuscularly injected into rats have been studied. Both "Imferon" (iron dextran) and "Ferrigen" (iron dextrin) induced a high incidence of sarcomas 9 to 14 months after commencement of the injections, whereas "Jectofer" (iron-sorbitol-citric acid) after the same length of time induced but a single tumour with the histological features of a fibroma. The results are discussed in the light of, among other factors, the amount of iron deposited and the differing molecular weights of the substances.

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THE RADIOACTIVE TRIOLEIN TEST IN MALIGNANT DISEASE

A. CENTI COLELLA,* W. S. REITH AND E. S. WILLIAMS

From the Institute of Nuclear Medicine, Middlesex Hospital Medical School, London, W.1

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REITH, WILLIAMS AND THOMAS (1961) have reported results using a standardised technique for the ^{131}I-triolein fat absorption test. In studying how the presence of diseases other than those involving fat malabsorption affected the results, it was observed that the level of blood radioactivity was below the normal range when malignant disease was present.

This observation has now been studied in 34 cases, brief details of these being given in Table I. Patients with tumours of the gastro-intestinal tract were excluded from the series. The age range was 13–89 years and there were 22 males and 12 females.

METHODS

Details of the preparation and standardisation of the labelled fatty meal are given by Reith *et al.* (1961). Thyroid uptake of ^{131}I was blocked by the administration of Lugol's solution on each of the two days preceding the test and on the day of the test the patient fasted overnight and ingested the labelled meal at about 9 a.m., and thereafter remained fasting until about noon, when food and fluid was allowed to be taken normally. Venous blood samples were taken at 2, 3, 4 and 6 hours after ingestion of the meal and faeces were collected 24 hourly until the faecal radioactivity had fallen approximately to background level.

The results of the tests were referred to those from a group of 8 normal subjects. The mean value of blood radioactivity at 2, 4 and 6 hours after ingestion of the meal was compared with the corresponding mean value in the whole malignant series, in the connective tissue tumour series, and in the carcinoma series, by applying Student's t test. A similar statistical comparison was made in a group of patients on whom the study was made both before treatment, and after tumour regression and clinical improvement had followed treatment.

RESULTS

The radioactivity contained in the total circulating blood in a group of normal subjects is shown in Fig. 1.

The broken line in Fig. 1 represents the mean circulating radioactivity and the shaded area one standard deviation on either side of the mean. Intercomparison between patients is difficult because of the irregular shape of the curves, so in each case the area enclosed by the curve, the abscissa, and the 6-hour ordinate was calculated and expressed as fraction of the similar area bounded by the curve of the mean normal plus one standard deviation (upper limit of the shaded area). This fraction was called the " relative manipulation curve area " (R.M.C.A.).

* Fellow of the International Atomic Energy Agency. Present address: Istituto di Radiologia Medica dell' Universita di Roma, Italy.

TABLE I.—*Experimental Data of the Malignant Series and of the Control Series*

Patients	Sex	Age	Activity of whole circulating blood as % of meal			R.M.C.A.
			2 hours	4 hours	6 hours	
Group of normals						
B	M.		18·2	14·2	11·8	83·4
C	,,		7·8	20·0	7·4	69·0
D	F.		24·7	15·6	15·4	104·2
E	M.		14·0	17·6	15·5	84·8
I	,,		18·7	14·7	13·9	87·2
N	F.		13·4	13·5	10·6	70·0
BF	,,		13·9	16·0	12·7	81·5
2BF	,,		10·7	16·0	12·1	74·6
Malignant neoplasms						
BY	F	44	1·3	6·4	7·3	24·5
CH	,,	49	9·1	9·5	8·4	48·6
CI	,,	69	8·6	14·0	9·3	65·8
CO	M	19	6·4	7·4	7·7	37·2
AC	,,	13	0·7	13·9	9·0	41·6
AE	,,	31	7·8	9·1	7·3	45·4
AN	,,	23	7·0	18·3	9·5	65·3
AI	,,	59	4·7	9·2	8·6	39·7
BA	F.	63	4·2	9·2	9·4	39·2
AF	M.	38	7·7	12·5	6·2	50·6
CT	,,	74	9·0	15·2	11·7	65·8
CU	,,	62	5·1	18·0	14·3	62·0
CX	,,	54	6·4	7·8	13·7	46·0
CY	,,	74	11·7	11·9	18·1	67·4
BW	F	78	9·5	10·5	10·1	54·5
BX	,,	66	4·6	6·3	5·8	29·8
CA	,,	74	8·1	8·6	8·2	45·3
BZ	,,	86	5·5	21·5	16·5	77·2
CC	M.	70	6·4	13·8	8·4	53·0
CD	F.	67	8·1	17·2	12·1	67·4
CF	,,	57	7·0	13·1	10·0	53·7
CG	,,	81	7·7	9·7	8·2	46·6
CK	M.	53	7·1	8·9	8·6	43·9
CL	F.	67	12·0	13·4	10·5	66·0
CM	M.	69	5·4	8·1	10·2	40·3
AX	,,	69	8·6	11·7	10·0	54·5
F	,,	71	10·0	10·3	7·7	42·4
AZ	,,	79	8·0	11·7	8·6	52·5
AV	,,	26	6·6	10·9	5·7	43·8
AY	,,	64	3·9	7·3	3·8	28·1
CP	,,	79	4·6	6·9	5·9	31·4
CQ	,,	64	2·7	9·3	11·4	38·5
CR	,,	46	7·3	8·4	8·2	43·6
CZ	,,	63	10·3	9·1	9·2	52·0

A histogram of the R.M.C.A. values of the patients studied in the present series is shown in Fig. 2. In all except three patients the faecal radioactivity is in the normal range suggesting that depression of the blood radioactivity curve is not due to defective absorption of fat.

Although eight patients in the malignant series had an R.M.C.A. value in the normal range statistical analysis of the difference between the mean of the malignant group and of the normal group gave the significance detailed in Fig. 3. The experimental data are recorded in Table I.

The malignant series was subdivided into (*a*) carcinomas and (*b*) other tumours and reticuloses and an analysis similar to that above was carried out, the result being shown in Fig. 4 and 5.

In 11 cases the test was carried out before and after the treatment. In 9 of these regression of the tumour and clinical improvement had occurred at the

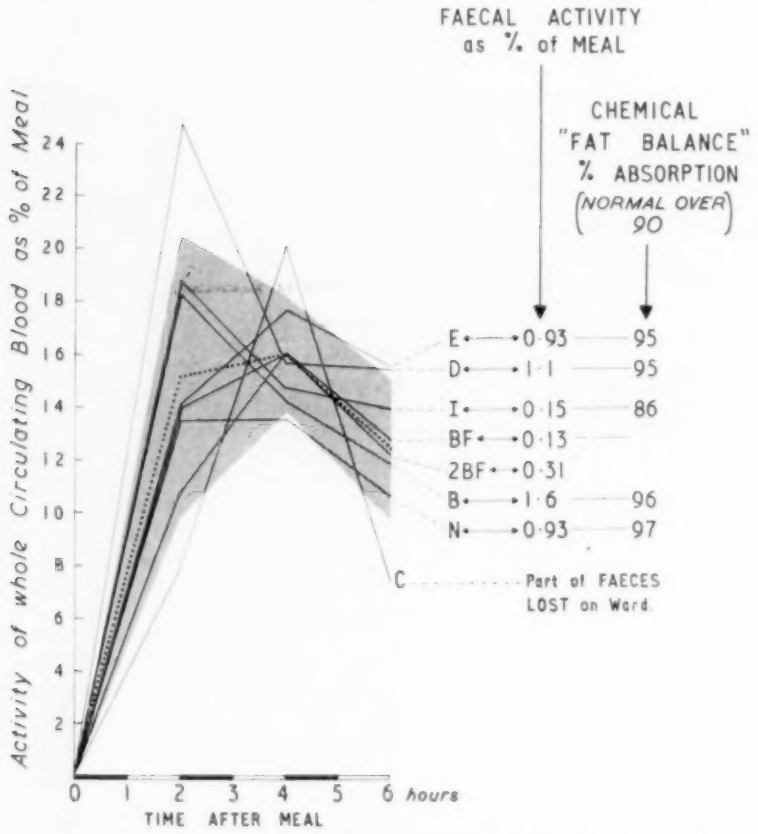

FIG. 1.—Blood radioactivity, faecal radioactivity, and results of chemical fat balance studies in a group of normal subjects.

time of the second test while in 2 cases the clinical condition had deteriorated. The data referring to these tests are shown in Table II and the comparison of the results is shown in Fig. 6.

We have not found any statistically evident influence on the R.M.C.A. values of the age of the patients (over 64 years and under), the rate of tumour growth, differentiation, or the tumour volume roughly calculated from clinical notes or X-ray evaluations.

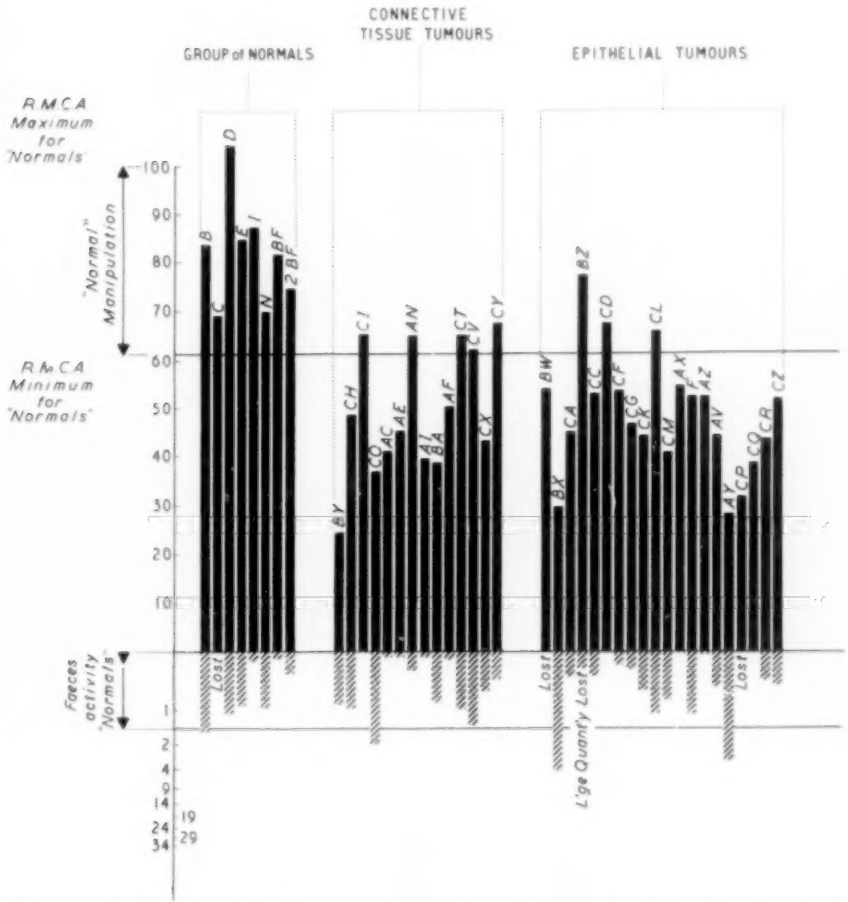

FIG. 2.—A histogram of the R.M.C.A. values and the faecal radioactivity obtained in a series of normal patients and in a series with malignant disease.

TABLE II.—*Experimental Data of Patients Tested Before and After Treatment*

Patients	Treatment	R.M.C.A. Before	R.M.C.A. After
BY	DXR-therapy	24·5	27·6
CF	Mastectomy	53·7	72·4
AC	DXR-therapy	41·6	57·7
CM	^{60}Co-therapy	40·3	46·8
CO	^{60}Co-therapy	37·2	51·6
CR	DXR-therapy	43·6	50·7
AE	^{60}Co-therapy	45·4	78·8
AF	^{60}Co-therapy	50·6	81·2
CH	Chemotherapy	48·6	46·8
BX	DXR-therapy	29·8	23·5
BA	Chemotherapy	39·2	26·4

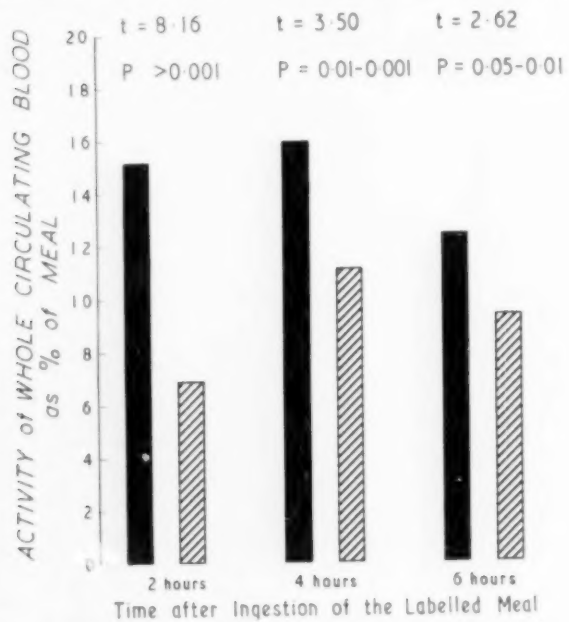

FIG. 3.—Histogram of the mean value of blood radioactivity of the control series (8 cases; black columns) and the malignant series (34 cases; shaded columns). P and t have the usual significance.

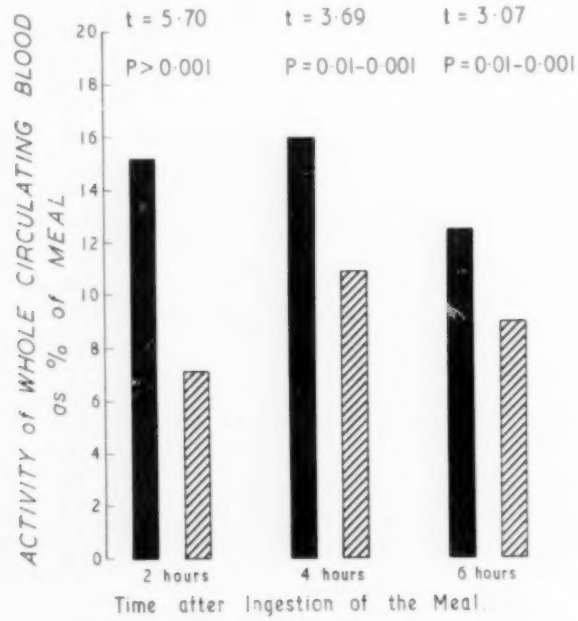

FIG. 4.—A comparison of controls with carcinoma cases.

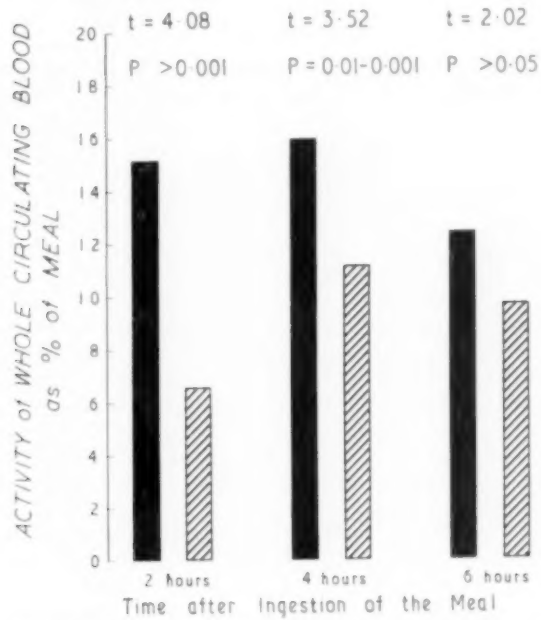

FIG. 5.—Comparison of controls with a group of reticuloses, leukaemias and sarcomas.

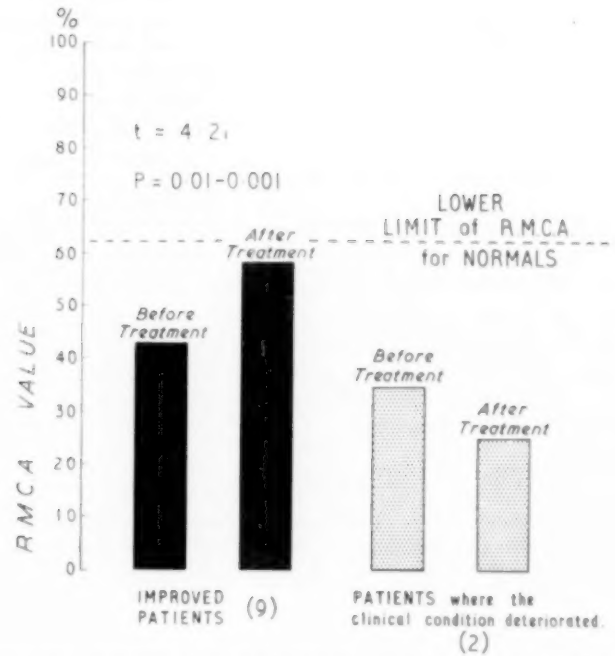

FIG. 6.—Comparison of the mean R.M.C.A. value of patients tested before and after treatment.

DISCUSSION

The technique used in this study is well known to be of limited utility and the results presented should be interpreted with caution. The significantly lower mean values of blood radioactivity found in the malignant series compared with that of the controls is apparently not due to a defect in absorption of fat. A dynamic state is reflected by the curve of blood radioactivity: it represents the balance between uptake of fat into the blood and its removal therefrom.

More rapid removal in malignant disease could merely be a secondary effect of cachexia analagous to that found in starvation. It could also be caused by preferential utilization by the tumour of freshly absorbed fat or by preferential utilization of triolein either directly or by mobilization.

More elaborate biochemical methods than that used here are necessary to throw further light on the problem but it appears to be well established that, in some species of animals, tumours utilize the fatty acids of the host. Medes, Thomas and Weinhouse (1953) calculated from the results of their experiments that only about 5 per cent of the fat content of tumours could be accounted for by endogenous synthesis. Haven (1940, 1941) has demonstrated the rapid turnover of tumour lecithin, cephalin, and sphingomyelin.

It is tempting to suppose that this extensive utilization of the host fat by tumours explains our findings, but a more simple explanation may be the correct one. Further work is certainly called for.

SUMMARY

1. The well known ^{131}I-triolein fat absorption test has been extended to study the blood radioactivity levels in a series of 34 cases of malignant disease.

2. Following the ingestion of the labelled fat the blood radioactivity was found to be lower in the malignant series than it was in the control series.

3. Successful treatment moved the curve of blood radioactivity towards the normal range while this effect did not occur in two cases where deterioration continued after treatment.

4. Possible explanations for the results are discussed.

Since this paper was submitted for publication, Gore and Popják (1961) state "that tumour cells obtain their lipids preformed from the host was experimentally confirmed".

Our thanks are gladly given to Professor Sir Brian Windeyer, Miss M. D. Snelling and Dr. A. M. Jelliffe for allowing us to study patients under their care, to Mr. D. H. Patey, Mr. R. S. Handley and Mr. J. H. L. Ferguson for allowing us to study patients before and after operation and to Mr. A. N. Goddard and Miss M.-T. Quellier for technical assistance.

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A FURTHER STUDY OF THE EFFECTS OF POLYCYCLIC HYDROCARBONS AND CROTON OIL ON MOUSE SKIN –SH LEVELS

G. CALCUTT

From the Department of Cancer Research, Mount Vernon Hospital and the Radium Institute, Northwood, Middlesex

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DURING a previous study of the effects of polycyclic hydrocarbons and croton oil on mouse skin sulphydryl (–SH) levels Calcutt and Coates (1961) found a time difference in the response to croton oil alone as compared with croton oil after a sensitising treatment with 7,12-dimethylbenzanthracene. This raised the question as to whether the sensitising dose of carcinogen had caused some tissue damage which had influenced the later response to croton oil.

The present work was designed to examine this possibility further and also to determine whether any similar effect was obtained when oleic acid was used as a promoting agent in the place of croton oil

All measurements of tissue –SH levels were made as in the previous work. Details of the technique are given by Calcutt and Doxey (1959) and Calcutt, Doxey and Coates (1960).

EXPERIMENTAL

One hundred and sixty Strong A male mice, all twelve weeks old, were divided into groups and treated as below :—

Group I—10 mice : untreated as controls.

Group II—30 mice : painted once with 0·2 ml. of acetone.

Group III—30 mice : painted once with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene in acetone.

Group IV—30 mice : painted once with 0·2 ml. of 0·05 per cent 7,12-dimethylbenzanthracene in acetone.

Group V—30 mice : painted once with 0·2 ml. of 0·025 per cent dimethylbenzanthracene in acetone.

Group VI—30 mice : painted once with 0·2 ml. of 0·1 per cent 3,4-benzopyrene in acetone.

All animals in Groups II to VI inclusive were then painted twice weekly with 0·2 ml. of 1·0 per cent croton oil in acetone. The treatments in all cases were to an area of approximately 1 cm. square in the middle of the back.

At daily intervals, commencing 24 hr. after the initial treatment, one animal was taken from each of Groups II to VI inclusive, killed and the total –SH content (protein bound plus glutathione –SH) estimated in the treated area of skin. At intervals during the course of the experiment one animal from Group I was killed and the total –SH content for a comparable piece of skin was measured.

The results in respect of Group I (control) animals showed only minor fluctuations in –SH levels so a mean and standard deviation were calculated. These

figures have been used as a base line for comparison with the results obtained in the experimental Groups. The findings for various experimental Groups are displayed in Fig. 1–5 inclusive.

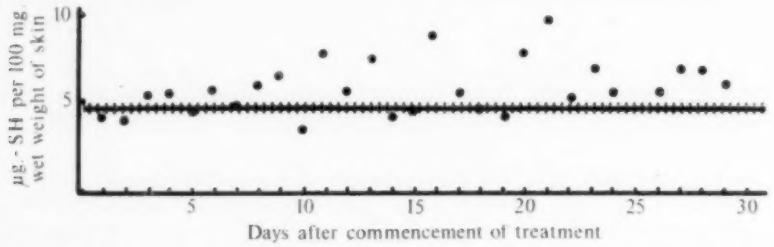

FIG. 1.—Skin –SH levels of mice painted once with acetone and then twice weekly with croton oil.

In this and all succeeding figures the mean control level is shown as a heavy line and the extent of the standard deviation by the hatched area. Experimental points are shown as heavy dots.

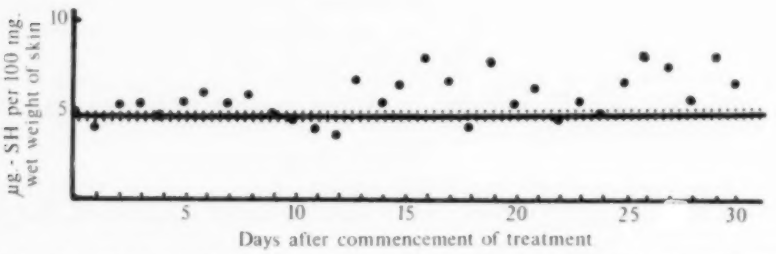

FIG. 2.—Skin –SH levels of mice painted once with 0·1 per cent 7,12-dimethylbenzanthracene and then twice weekly with croton oil.

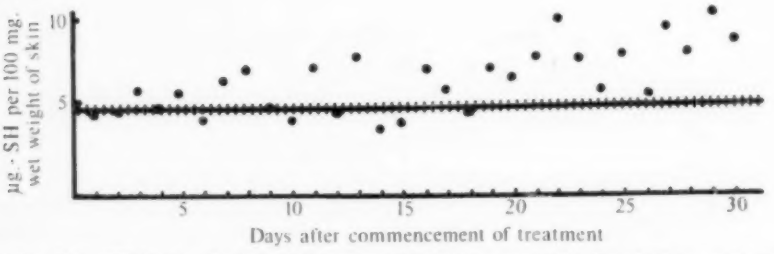

FIG. 3.—Skin –SH levels of mice painted once with 0·05 per cent 7,12-dimethylbenzanthracene and then twice weekly with croton oil.

Treatment with croton oil after a single painting with acetone has shown an almost immediate rise in skin –SH levels above the normal value. This picture is identical with that obtained previously by Calcutt and Coates (1961) when croton oil was painted on mouse skin, but without the initial painting with acetone.

Inspection of Fig. 2–5 shows that there is no essential difference between the cases initially treated with a carcinogen and the series without the carcinogen. The figures previously obtained by Calcutt and Coates (1961) showing a delayed response to croton oil after treatment with 7,12-dimethylbenzanthracene would appear to be fortuitous and as such must be disregarded.

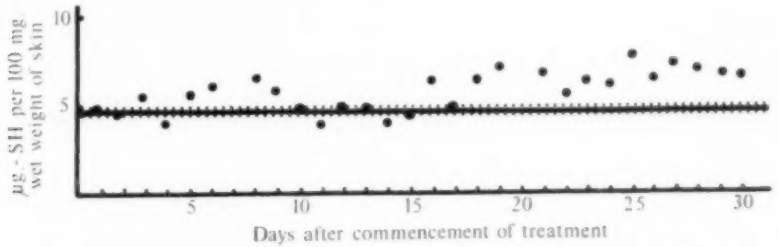

FIG. 4.—Skin –SH levels of mice painted once with 0·025 per cent 7,12-dimethylbenzanthracene and then twice weekly with croton oil.

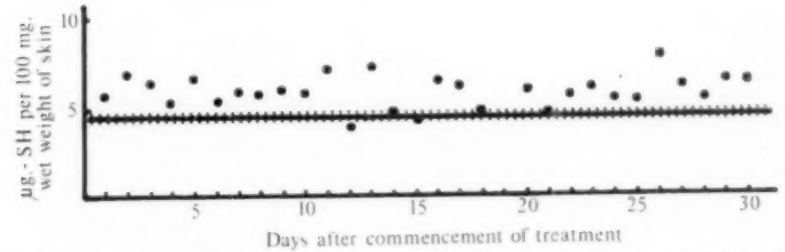

FIG. 5.—Skin –SH levels of mice painted once with 0·1 per cent 3,4-benzopyrene and then twice weekly with croton oil.

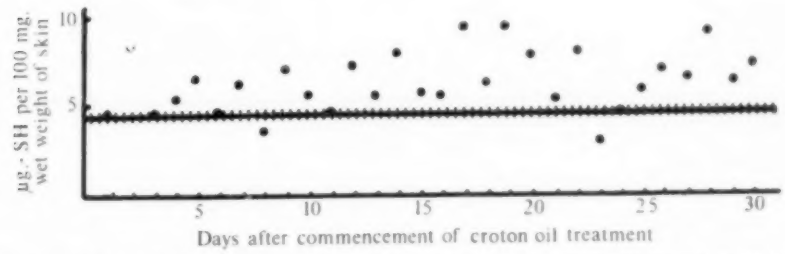

FIG. 6.—Skin –SH levels of mice painted twice weekly with croton oil commencing 37 days after a single painting with acetone.

These experiments have been extended to cover the situation where there is a delay between the application of a sensitising dose of carcinogen and the subsequent promotion of tumour formation by croton oil treatment.

Seventy four Strong A male mice were divided into groups of 14, 30 and 30. The fourteen were used as untreated controls whilst one group of thirty were

treated with 0·2 ml. of acetone per animal and the remaining thirty were each treated with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene in acetone. After a delay period of thirty seven days treatment of the two experimental groups with croton oil was started. Each animal received a 0·2 ml. of 1·0 per cent croton oil in acetone twice weekly over the initially treated area. From the commence-

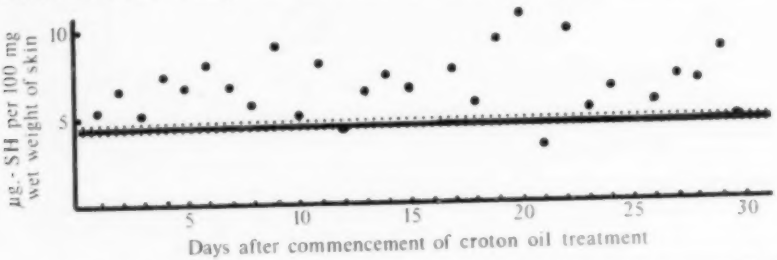

FIG. 7.—Skin –SH levels of mice painted twice weekly with croton oil commencing 37 days after a single painting with 7,12-dimethylbenzanthracene.

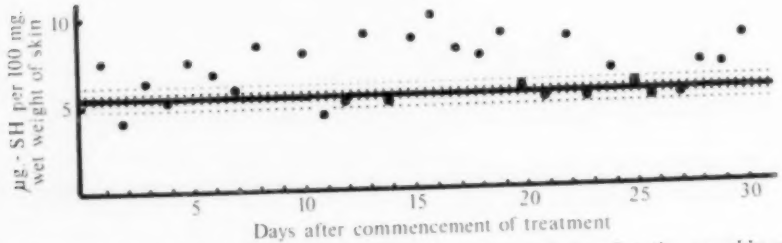

FIG. 8.—Skin –SH levels of mice painted once with acetone and then five times weekly with oleic acid.

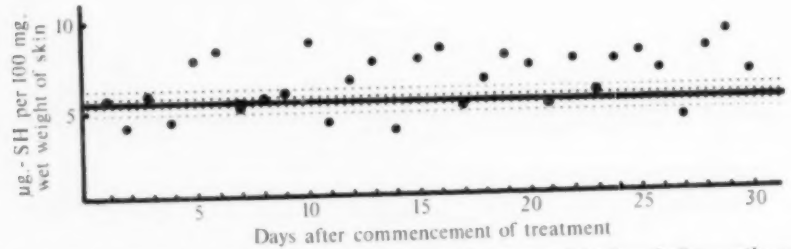

FIG. 9.—Skin –SH levels of mice painted once with 0·1 per cent 7,12-dimethylbenzanthracene and then five times weekly with oleic acid.

ment of this period one animal of each group was killed daily and the –SH level of the treated area of skin was measured. During the course of the experiment the control animals were killed and –SH measurements made on similar skin areas.

As previously a mean control value and standard deviation were calculated. The experimental results are displayed against these values in Fig. 6 and 7.

Again it is apparent that pretreatment with the carcinogen has not affected the timing of the later response to croton oil treatment.

Further consideration has also been given to this problem in respect of the use of oleic acid as a tumour promoting agent. It has already been shown by Calcutt (1961) that this agent will induce a rise in mouse skin –SH levels in a similar fashion to croton oil.

Seventy two Strong A male mice, aged fourteen weeks, were divided into groups of 12, 35 and 35. The group of 12 were used as controls. One group of 35 were each painted once with 0·2 ml. of 0·1 per cent 7,12-dimethylbenzanthracene in acetone, and each animal of the remaining group was painted once with 0·2 ml. of acetone. The animals of both experimental groups were then painted five times weekly over the pretreated area with redistilled oleic acid. Daily –SH measurements were made in respect of the experimental groups and the control values were determined at intervals during the course of the experiment.

For the control –SH values a mean and standard deviation were calculated. These figures were used as a base line for the display of the experimental values (Fig. 8 and 9). As in the previous experiments no essential difference was determined between the two experimental groups.

DISCUSSION

From the foregoing results it is apparent that a sensitising dose of a carcinogen does not influence the timing of the response of the skin to subsequent applications of cocarcinogen. The result previously found by Calcutt and Coates (1961) must therefore be disregarded. Additionally, inspection of the diagrams also suggests that there is no distinction between the initial treatments and the degree of response, i.e. the extent of the increase in –SH values, obtained by subsequent treatment.

In more general terms, this lack of influence of the sensitising treatment on the biochemical response to the tumour promoting treatment would suggest that at least two completely independent cellular mechanisms are involved. In this event the case of tumour promotion by repeated treatment with a carcinogen becomes interesting in that the implication of cellular activity at two separate sites by the carcinogen becomes apparent. This question will be considered in more detail in a future paper.

SUMMARY

1. It has been found that variations in dosage or carcinogen do not affect the timing of later rises in mouse skin –SH levels due to croton oil or oleic acid.

2. The extent of the changes in skin –SH values induced by treatment with cocarciongens is not influenced by the dosage of the sensitising agent.

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THE EFFECT OF TUMOURS, OF LEUKAEMIA, AND OF SOME VIRUSES ASSOCIATED WITH THEM, ON THE PLASMA LACTIC DEHYDROGENASE ACTIVITY OF MICE

D. H. ADAMS, K. E. K. ROWSON, AND M. H. SALAMAN

From the Cancer Research Department, London Hospital Medical College, London, E.1

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IT is now well established that the plasma lactic dehydrogenase (PLDH) activity is raised in animals bearing a variety of spontaneous or transplanted tumours, and in tumour-bearing patients (e.g. Hill and Levi, 1954; Hsieh, Suntzeff and Cowdry, 1956; Manso, Sugiura and Wroblewski, 1958; Wroblewski, 1959; Hill and Jordan, 1957). Riley and Wroblewski (1960) have also shown that PLDH rises and falls in mice during tumour growth and regression respectively. Recently Riley, Lilly, Huerto and Bardell (1960) showed that plasma taken from tumour-bearing mice, when injected into normal mice, induced a rapid and prolonged rise in PLDH to 5–10 times the normal level, beginning within two days and lasting for at least 70 days. This effect on PLDH activity was serially transmissable by mouse plasma, and the responsible agent passed a bacteria-proof filter. According to these authors mice bearing a wide variety of transplantable tumours, and mice in which leukaemia had been induced by the Friend or Moloney viruses, gave similar results. Apart from the intrinsic interest of these observations, the possibility that PLDH levels might prove a sensitive early assay for the presence of leukaemogenic and tumour-producing viruses induced us to take up the problem.

Although it soon became evident that measurements of PLDH activity would not serve as such an assay method, results of general interest were obtained, and are reported below.

MATERIALS AND METHODS

Animals.—Mice of the CBA and AKR inbred strains were bred by brother-sister mating in this laboratory.

Mice of a heterogeneous stock albino strain were also used (obtained from Messrs. Schofield, Oldham, Lancs.), and bred by random mating in this laboratory.

The mice were fed on commercial cubes (Diet 41B) and water, both *ad libitum*, with a twice-weekly supplement of rolled oats.

Tumours.—Sarcoma 37 (S37) was obtained originally from the Imperial Cancer Research Fund Laboratories, Mill Hill, and maintained in this laboratory by serial passage in CBA mice. Sarcoma 180 (S180) in solid form was obtained from the Chester Beatty Research Institute, and in ascitic form from the Central Public Health Laboratory, Colindale.

Solid tumours were minced, and fragments implanted subcutaneously with a Bashford needle.

Spleen, thymus, and lymph nodes of leukaemic mice were pressed through a nylon mesh (22 threads/cm.) into isotonic sodium chloride solution, and the resulting suspensions injected intraperitoneally.

Ascitic fluids were also injected intraperitoneally.

Virus preparations.—Moloney's leukaemogenic virus was originally derived from S37 (Moloney, 1960). A lyophilysed preparation of this agent was kindly given to us by Dr. J. B. Moloney. This was reconstituted in distilled water and stored at −70° C. until required. Some was used to infect mouse embryo tissue cultures (Salaman, Rowson and Harvey, 1961) grown in Earle's medium with 0·5 per cent lactalbumen hydrolysate and 5 per cent horse serum. After one, and again after three, blind passages, at 10–14 day intervals, the tissue culture fluid and homogenised cells were stored and used as a source of virus (TCP Moloney virus).

Polyoma virus (strain LHP1), originally obtained in this department from AKR leukaemic tissues (Salaman, 1959), was propagated in mouse embryo tissue cultures (Salaman and Rowson, 1959, 1960).

All virus preparations were inoculated intraperitoneally.

Plasma for injection.—Blood was obtained from the severed brachial artery, or from the heart, under ether anaesthesia, heparinised, centrifuged, and the plasma injected intraperitoneally.

Test groups.—For all inoculations groups of 4–6 mice were used.

Blood samples for biochemical estimation.—Blood (0.05 ml.) was taken from the cut end of the tail into a pipette, and then diluted with isotonic saline containing 10 units/ml. of heparin. Dilutions varied from 1 : 6 to 1 : 24 according to the anticipated enzyme level. The suspension was centrifuged (1000 × **g** for 10 minutes) and the supernatant very carefully removed with a Pasteur pipette. Samples showing any haemolysis were discarded, but this occurred only rarely. After removal of the plasma for PLDH estimation the cells were resuspended and pooled; they were then recentrifuged in an Orpwood-Price haematocrit tube at 1000 × **g** for 10 minutes to measure the packed cell volume.

Estimation of PLDH activity.—PLDH was estimated by measuring the disappearance of pyruvate during 30 minutes at 37° C., as described in the Sigma Chemical Company's Technical Bulletin No. 500 (Berger and Broida, 1960) with the following modifications. Because of the difficulty of obtaining sufficient blood from the tail, half the recommended quantities of plasma, substrate, colour reagent, and 0·4 N NaOH were used. For the same reason the blood was diluted before separation of the plasma from the red cells, the average plasma volume being calculated from the haematocrit reading. In our hands the recommended quantity, 1 mg/ml., of diphosphypyridine nucleotide coenzyme did not yield maximum activities, which required 1·5 mg/ml. After colour development the solutions were placed in a "Spekker" colorimeter, using a green filter, and the pyruvate disappearance measured by comparison with a standard curve, as described in the Sigma Bulletin, the results being given in "Berger & Broida" (BB) units.

RESULTS

Homologously transplantable tumours

In separate experiments Sarcoma 37 and 180, obtained from the two different sources already mentioned, were inoculated into stock albino mice, and their

PLDH activity was measured at intervals. Essentially similar results were obtained with these tumours, and only those obtained with S37 are reported below. As shown in Fig. 1*a* there was a rapid rise in PLDH from 400 to about 4000 units in three days. The level remained unchanged for a further 4 days and then rose again steeply. This second phase corresponded with the development of a subcutaneous tumour.

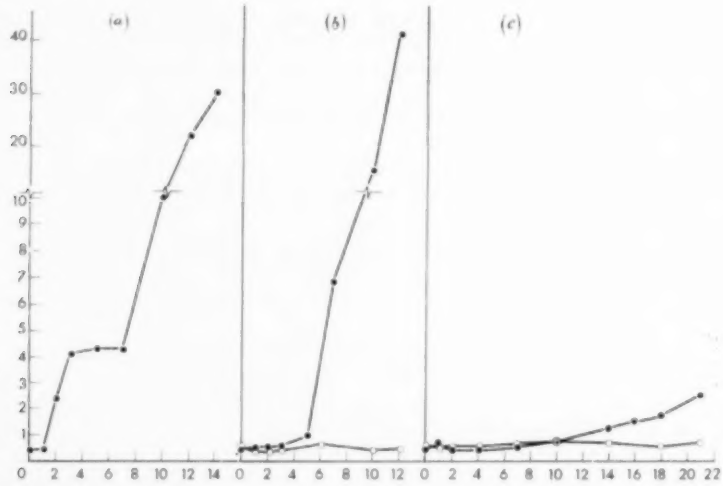

FIG. 1.—Plasma lactic dehydrogenase (PLDH) levels in mice after inoculation with various tissues.

(*a*) Sarcoma 37 into stock albinos.
(*b*) ● —— ● AKR leukaemic tissue into AKRs.
○ —— ○ Ditto into stock albinos.
(*c*) ● —— ● CBA leukaemic tissue, induced by TCP Moloney virus, into CBAs.
○ —— ○ Liver + spleen + kidney tissue from normal CBAs into CBAs.

In this and subsequent figures curves are drawn through the average values for each group of mice (six per group except where otherwise stated). Where the scale permits, individual points represent the values for each mouse.
Ordinates: Plasma lactic dehydrogenase activity (BB units $\times 10^{-3}$).
Abscissae: Days.

Transplantation of spontaneous and virus-induced leukaemias

When a suspension of lymphatic tissue from a spontaneously leukaemic AKR mouse was injected intraperitoneally the result depended on the type of recipient mouse. AKR mice, in which the leukaemic cells grew, showed no change in PLDH activity until about the 6th day, but then there was a continuous rise up to a level of 40,000 units by the 12th day (Fig. 1*b*), which accompanied the development of leukaemia. Stock albinos, in which the leukaemic cells did not grow, showed no change in PLDH activity (Fig. 1*b*).

An essentially similar result was obtained when a lymphatic tissue suspension from a CBA mouse in which leukaemia had been induced by TCP Moloney virus was inoculated into CBA mice (Fig. 1*c*): there was no early rise in PLDH, such

as had been seen after implantation of S37, but a rise after the 10th day, when signs of leukaemia began to appear. This leukaemia grew more slowly than the AKR leukaemia, and the rise in PLDH was slower and did not reach such a high level.

A control group of CBA mice inoculated intraperitoneally with a suspension of mixed normal CBA liver, spleen, and kidney tissue showed no change in PLDH activity (Fig. 1*c*).

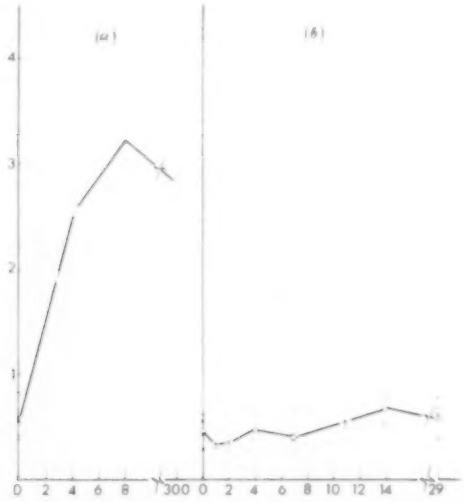

FIG. 2.—PLDH levels in stock albinos inoculated with Moloney virus.
(*a*) Moloney virus as originally obtained from Dr. Moloney.
(*b*) Ditto after tissue culture passage.
Ordinates: Plasma lactic dehydrogenase activity (BB units $\times 10^{-3}$).
Abscissae: Days.

Inoculation of viruses

(a) *Moloney virus.*—The original sample obtained from Dr. Moloney, when injected into adult stock albinos, produced a rapid rise in PLDH (Fig. 2*a*). But after one, or three, passages through tissue cultures, injection produced no early change in PLDH activity (Fig. 2*b*).

In order to follow PLDH activity during leukaemogenesis by Moloney virus, newborn mice were used. Eleven stock albinos less than 24 hours old were injected with TCP virus, and their PLDH measured at intervals after weaning. No rise was detected until they were 8 weeks old, when two showed a definitely raised level. At this time all appeared healthy, but two weeks later these two, and three others, became obviously leukaemic. Thus in the development of leukaemia due to Moloney virus, as in that of transplanted leukaemia, a rise in PLDH only slightly antedates visible signs of disease.

(b) *Polyoma virus.*—Though mice which had been inoculated in infancy with polyoma virus, and bore large parotid tumours, had a high PLDH activity,

injection of the virus into adult mice (with no haemaglutination-inhibiting antibodies in their serum) did not produce a rise in PLDH.

Detection of a transmissable agent which causes a rapid rise in PLDH activity

The fact that inoculation of S37 (the strain maintained in this laboratory) and of Moloney virus (the sample obtained from Dr. Moloney, and derived by him

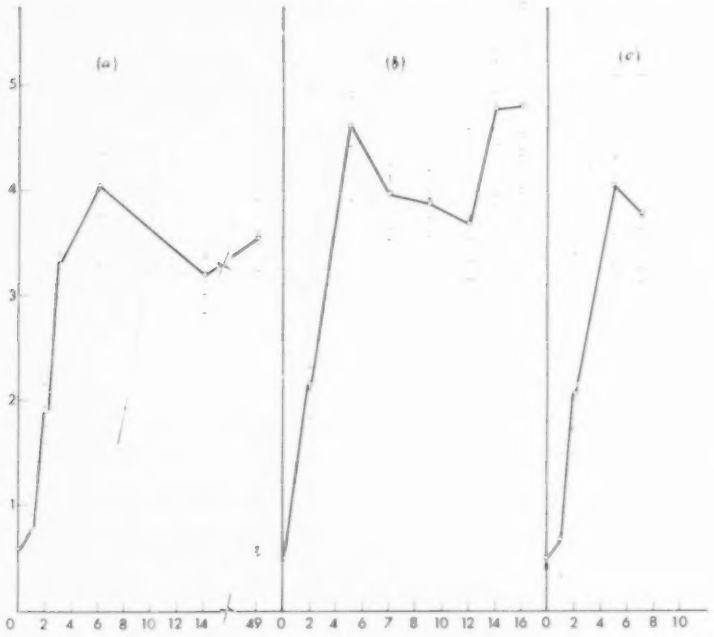

FIG. 3.—PLDH activities in stock albino mice inoculated with plasma:—
(*a*) from mice bearing Sarcoma 37.
(*b*) from mice after two serial plasma passages from mice bearing Sarcoma 37.
(*c*) from mice inoculated with original Moloney virus.

Ordinates: Plasma lactic dehydrogenase activity (BB units $\times 10^{-3}$).
Abscissae: Days.

from his strain of S37) both induced a rapid rise in PLDH similar to that reported by Riley *et al.* (1960) suggested that the transmissable agent which they described was present in these inocula.

Plasma from S37-bearing mice when injected into stock albinos produced a rise of PLDH within 48 hours, and the level remained high (3000–4000 units) thereafter (Fig. 3*a*). The curve is similar to the first part of the biphasic response to inoculation of S37 tissue (i.e. before tumour growth is palpable) (Fig. 1*a*). Plasma from the inoculated mice in turn produced a similar effect on passage to other mice (Fig. 3*b* shows the 3rd passage).

Plasma of mice inoculated with the original sample of Moloney virus, when injected into other mice, also produced a rapid rise in PLDH (Fig. 3c).

Plasma of mice inoculated with AKR spontaneous leukaemic tissue suspension produced no change in PLDH activity of mice into which it was injected.

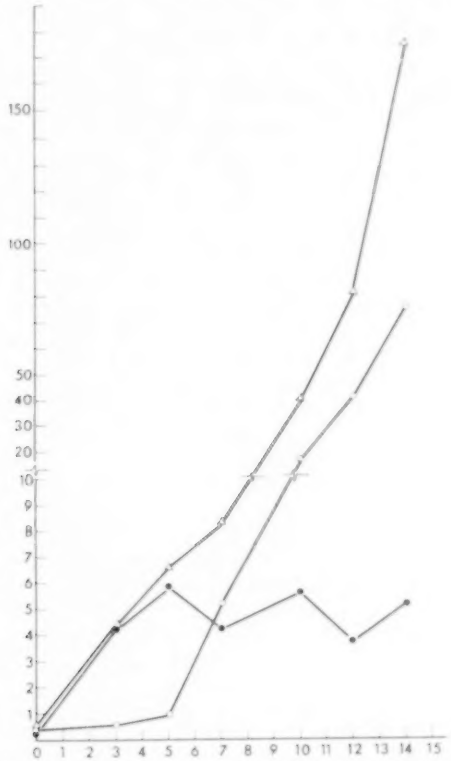

FIG. 4.—PLDH activities in AKR mice inoculated with

● —— ● Riley agent
○ —— ○ AKR leukaemic tissue
△ —— △ Riley agent + AKR leukaemic tissue.

Ordinates: Plasma lactic dehydrogenase activity (BB units $\times 10^{-3}$).
Abscissae: Days.

The combined effect of inoculation of Riley agent and leukaemic cells

An experiment was designed to discover whether the early and sustained rise in PLDH due to the transmissible virus obtained from S37 and the later rise in PLDH due to the growth of neoplastic tissue were additive phenomena or not. Of three groups of 6 AKR mice, one was inoculated with plasma from Riley agent-infected mice, one with AKR leukaemic cells, and a third with both plasma and cells. The PLDH levels of each group are shown in Fig. 4. For about the first 7 days the level in the doubly-inoculated group is approximately the sum of the

levels in the other two groups, but later there is a suggestion of synergic action, for the PLDH level in the doubly-inoculated group is much higher than would be expected if the two factors were working independently.

DISCUSSION

The biphasic rise in PLDH (Fig. 1*a*) following the inoculation of S37 is similar to that reported by Riley and Wroblewski (1960) following the injection of Ehrlich carcinoma cells. The presence in the plasma of S37-bearing mice of a transmissible agent capable of reproducing the first part of the curve is in agreement with the report of Riley *et al.* (1960), who were able to demonstrate the presence of an "enzyme-elevating" agent in the blood of mice bearing various transplanted tumours, and of eight mice with spontaneous mammary carcinomata (presence of Bittner agent not stated). However, in the present study, plasma of mice with spontaneous leukaemia, or leukaemia due to tissue culture-passaged Moloney virus infection, were not found to carry a virus of the Riley type. The original freeze-dried preparation of Moloney virus obtained from Dr. Moloney contained such an agent, but it was lost after tissue culture passage (Fig. 2*a* and 2*b*), while the leukaemogenic potency appeared actually increased by this process (Salaman, Rowson and Harvey, 1961).

It is clear from this result, as from that of injecting AKR leukaemic cells, that simple measurements of PLDH levels will not serve as an early indication of infection by leukaemogenic viruses.

Since the transplantation of a tumour inevitably involves the passage of some plasma it is not possible to say at present whether, in those cases where transplantation is followed by an early rise in PLDH, the Riley virus is actually carried in the tumour cells. Since a spontaneous leukaemia, when inoculated into mice, did not produce this early effect, it is evident that the Riley virus does not invariably accompany neoplastic tissue.

PLDH levels in AKR mice inoculated with both Riley virus and tissue suspensions from spontaneous AKR leukaemia were approximately the sum of those in mice inoculated with each separately, for the first week. Later the effect of the combined inocula rose to more than double that of the sum of the effects of each. This synergism suggests *prima facie* a linked activity. Further study of this phenomenon will include an attempt to discover the site of origin of the PLDH which each agent releases. The possibilities that the Riley agent accelerates the growth of tumours or of leukaemia, or increases the rate of loss of enzyme from neoplastic cells, will be considered.

It is a remarkable fact that PLDH level once elevated by injection of Riley virus, remains elevated for many months, and perhaps for life. No less remarkable is that the virus is demonstrable in the plasma for similar periods. Apart from their intrinsic interest these observations throw a warning light on the field of study of tumour-host relations. It is possible that some effects on the host now ascribed to tumour growth could be due to passage of viruses along with tumour inocula.

SUMMARY

1. Inoculation into mice of the homologously transplantable tumours S37 and S180 resulted in an early rise in plasma lactic dehydrogenase (PLDH)

activity, followed by a further rise during the period of visible growth. Plasma from these mice transmitted the early effect on PLDH activity to other mice in series.

2. Inoculation into AKR mice of lymphatic tissue suspensions from spontaneous AKR leukaemia resulted in a rise in PLDH at the time of development of clinical leukaemia. In stock albino mice these suspensions produced no change in PLDH activity, and no leukaemia.

3. A preparation of Moloney virus (derived from S37 by Dr. Moloney, and obtained from him) produced an early rise in PLDH on injection into adult mice, similar to that produced by suspensions of S37.

4. Moloney virus after passage through mouse enbryo tissue cultures (TCP) had lost its power of rapidly raising PLDH, but retained its leukaemogenic activity.

5. Mice injected when newborn with TCP Moloney virus showed no rise in PLDH until shortly before the appearance of clinical leukaemia. Plasma of the leukaemic mice did not produce a rise of PLDH on injection.

6. Polyoma virus injected into polyoma-free adult mice, did not alter PLDH activity, but mice bearing large polyoma-induced parotid tumours had high PLDH levels.

7. The PLDH levels of AKR mice inoculated with both tissue suspensions from spontaneous AKR leukaemia and plasma from an S37-bearing mouse rose to more than twice the sum of the levels attained by the PLDH in mice inoculated with each separately.

8. It is concluded that :

(*a*) a transmissible agent which causes a rapid and sustained rise in PLDH is present in some homologously transplantable tumours,

(*b*) the high PLDH activity of leukaemic mice is not necessarily associated with the presence of this agent, and

(*c*) at least one tumour-producing virus (polyoma) and one leukaemogenic virus (Moloney) do not have a similar action.

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PLASMA β-GLUCURONIDASE AS AN INDEX OF HORMONE DEPENDENCY OF BREAST TUMOURS

B. L. WHITAKER

From the Royal Free Hospital, London, W.C.1

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MANY of the alcohols, phenols and carboxylic acids of mammalian tissue exist in combination with D-glucuronic acid as β-D-glycopyranosiduronic acids. This applies to the steroid hormones and also to various substances, such as stilboestrol, which are administered therapeutically. The effect of such conjugation is usually to reduce the biological activity of the substance (Bray, 1953; Teague, 1954).

The hydrolysis of such biosynthetic β-D-glucopyranosiduronic acids to D-glycuronic acid and aglycons is catalyzed by the enzyme β-glucuronidase which is under endocrine control (Levvy, 1953).

Kerr and Levvy (1947) and Levvy, Kerr and Campbell (1948) demonstrated a connection between the level of β-glucuronidase in the tissues and processes of growth and repair.

Goldbarg, Pineda, Banks and Rutenberg (1959) and Whitaker (1960) observed raised β-glucuronidase levels in the blood of a proportion of patients with breast cancer. In 47 cases investigated an abnormally high β-glucuronidase was observed in approximately 50 per cent (Whitaker, 1960).

The remission rate following hypophysectomy for metastatic breast cancer has been estimated by various workers (Luft, Olivecrona, Ikkos, Nilsson and Ljunggren, 1956; Baron, Gurling and Radley-Smith, 1958) as between 45 and 50 per cent.

The present investigation was undertaken in order to establish whether or not there was any correlation between the plasma β-glucuronidase and the remission rate following hypophysectomy.

MATERIAL

Forty women and one man suffering from metastatic breast carcinoma were studied. Of these thirty-four were submitted either to open craniotomy or stereotaxic implantation of radioactive material into the pituitary with the object of obtaining complete destruction of the pituitary. This object was achieved in twenty-eight cases, but in six the operation was abandoned for technical reasons or the implant did not produce destruction of the pituitary. These six cases are included as a control group.

The remaining seven cases have been included to illustrate certain effects of hormone therapy on the plasma β-glucuronidase.

METHOD

The method of estimation of the plasma β-glucuronidase has been that of Tallalay, Fishman and Huggins (1946), modified by Boyland, Wallace and Williams (1955).

The normal range of plasma β-glucuronidase using this method was taken as 0·96–6·20 units, being ± two standard deviations about a mean of 3·58 units. This range was obtained by estimation of the plasma levels of fifty normal women whose age and menstrual status were listed in a previous communication (Whitaker, 1960).

Six aspects of the clinical histories of the forty-one patients in this series have been studied in relation to plasma β-glucuronidase; namely, the effects of androgens, oestrogens and cortisone, the relationship of the pre-operative β-glucuronidase levels to the response to hypophysectomy, the effect of hypophysectomy itself on the enzyme, and the relationship of the enzyme to the advance of the disease process.

(1) *The effect of androgen administration*

Estimations of plasma β-glucuronidase were made in nineteen cases during or after the cessation of androgen therapy. In five the androgens were started while the patient was under observation and in the remainder androgens had been administered until the time of admission to hospital, when the treatment was stopped.

Group 1, *Table I.*—Of the five cases who started androgens while under observation three received methyl testosterone by mouth, one testosterone propionate by injection, and one case received both in succession.

In four of the five cases single pre-therapy observations were made, and in one eight were obtained.

In three cases testosterone was stopped while the patient was still under observation, and in column 5, Table I are given the mean β-glucuronidase values for each case for the three days following, but not including, the day on which the drug was stopped. In each case the level of β-glucuronidase continued to rise for some days after withdrawal of the drug and the maximum level reached before any subsequent fall is given in column 7, Table I. The other two cases were discharged to terminal homes, still taking testosterone, and in their case the figures in column 7, Table I represent the last reading before discharge.

Two of the cases who received methyl testosterone showed a sudden sharp rise in β-glucuronidase, as illustrated in Fig. 1 (case 91). After withdrawal of the hormone the β-glucuronidase fell rapidly in each case. Both were subjected to hypophysectomy and both achieved a major remission. The third case received a smaller dose of methyl testosterone (5 mg. t.d.s.) and at the time of discharge five days later the β-glucuronidase had risen by 1·31 units.

Case 39, unlike the other four cases, had a greatly elevated pre-therapy reading (13·9 units). She was treated at first with injections of 100 mg. testosterone propionate intramuscularly on alternate days for eight doses. Following an initial peak of 18·9 units the β-glucuronidase returned gradually to its original level by the time the eighth dose had been given. For the next seven days she received methyl testosterone by mouth, 20 mg t.d.s., with a further fall of β-glucuronidase to 10·2 units. At this time she had a sudden increase in pain from bone deposits and the treatment was altered to 200 mg. testosterone propionate intramuscularly on each of the next three days. Coincident with this there was a rise of β-glucuronidase to 19·4 units, though whether due to the increase androgen or to advance of the disease is not clear. This patient did not respond to hypophysectomy.

Case 105 received intramuscular testosterone propionate 100 mg. on alternate days. After 11 days the β-glucuronidase had fallen by 2·1 units.

TABLE I.—*Androgen Therapy While Under Observation*

Case Number	Mean pre-androgen β-glucuronidase	Number of estimations	Type, dose and duration of androgen	Mean β-glucuronidase for three days following end of androgen therapy	Number of estimations	Maximum post-androgen reading	Subsequent rise or fall	Interval	Response to hypophysectomy
91	7·00	1	Methyl testosterone 15 mg. t.d.s. 10 days	15·52	2	16·74	Fall to 2·65	16 days hypo. intervening	Remission
39	13·90	1	Testosterone propionate 100 mg. a.d. for 16 days then methyl test. 20 mg. t.d.s. for 7 days then T/P 200 mg. daily × 3	18·60	2	19·40	Slight fall to 17·80	3 days	None
74	5·79	8	Methyl testosterone 5 mg. t.d.s.	Discharged, still on androgens	—	7·10 after 5 days	—	Not withdrawn	Not performed
89	4·33 (actually taken on day 2)	1	Methyl testosterone 150 mg. on day 1, then 15 mg. t.d.s. for 11 days	9·12	2	12·42	Fall to 5·53	6 days	Remission
105	6·34	1	Testosterone propionate 100 mg. a.d.	Discharged, still on androgens	—	4·24 after 11 days	—	Not withdrawn	Not performed

TABLE III.—*Ethinyl Oestradiol Therapy While Under Observation*

Case Number	Pre-oestrogen β-glucuronidase	Number of readings	Dose and duration	Mean β-glucuronidase for 3 days post-oestrogen	Number of readings	Maximum post-oestrogen	Response to hypophysectomy
14	3·71	5	5 mg. t.d.s., 7 days	6·9	1	6·9	Group I
42	2·55	4	2 mg. daily, 4 days	—	—	5·72 (3rd day of course)	Pituitary not destroyed
73	8·40	5	5 mg. t.d.s., 12 days	21·51	2	23·22	—
94	4·95	4	1 mg. t.d.s. for 3 days, then 0·5 mg. b.d. for 11	6·03	2	9·25	Group IV
108 (male)	6·74	3	1 mg. t.d.s. not withdrawn	—	—	10·60 after 21 days	—
100	7·20	3	2 mg. t.d.s., 4 days	15·0	1	15·01	Group IV

TABLE II.—*Androgen Therapy Until Admission*

Case Number	Initial reading β-glucuronidase	Interval since androgens	Last reading before other treatment	Interval (days)	Type, dose and duration of androgen therapy	Response to hypophysectomy
(i) *Oral Methyl Testosterone :*						
7	11·20	1 day	5·45	9	Meth. test. 15 mg. t.d.s. 6 weeks	Group III
13	13·40	5 days	4·20	17	Meth. test. 15 mg. t.d.s.	Pituitary function not abolished
14*	9·05	1 day	2·60	10	Meth. test. 5 mg. t.d.s. 1 month	Group I
16	8·70	16 days	4·80	34	Meth. test. 15 mg. t.d.s. 1 year	Group I
81	23·5	1 day	12·60	7	Meth. test. 15 mg. t.d.s. 1 month	Group IV
100	9·80	1 day	10·50	20	Meth. test. 15 mg. t.d.s. 3 months	Group IV
(ii) *Testosterone propionate by intramuscular injection :*						
9	3·92	Androgens continued until op.	3·55	48	Test. prop. 200 mg./day 56 days	Pituitary function not abolished
14*	2·60	2 days	—	—	Test. prop. 100 mg. daily 5 days	Group I
88	4·40	1 day	5·32	3	Test. prop. 100 mg. daily 7 months	Group I
103	6·20	7 days	4·56	22	Test. prop. 6 months	Group IV
(iii) *Non-virilizing Androgens :*						
23	2·21	5 days	3·10	7	Androstalone 25 mg. t.d.s. for 4 yr then 50 mg. t.d.s. for 3 months	Group I
28	5·05	1 day	—	—	Durabolin 3 months	—
106	10·70	1 day	10·12	14	Androstalone 25 mg. t.d.s. 2 months	Pituitary function not abolished
107	6·85	1 day	6·70	6	Durabolin 25 mg. weekly 6 months	Pituitary function not abolished

* After withdrawal of oral methyl testosterone from Case 14 her β-glucuronidase fell over the next ten days to 2·6 units. *During* this ten days she was given intramuscular testosterone propionate.

Group 2, Table II —In this group of fourteen cases six had had oral methyl testosterone, four intramuscular testosterone propionate and four non-virilizing androgens in the form of " Durabolin " (norandrostenolone phenyl propionate) in two cases and " androstalone " (methyl androstanolone) in the other two. In each of the fourteen the androgen was discontinued just before admission.

In Column 4, Table II, are given the last available figures for β-glucuronidase following withdrawal of androgens and before the start of any other hormonal treatment.

(i) In the six cases who had had oral methyl testosterone the maximal interval between cessation of androgens and the first β-glucuronidase assay was sixteen days. This case showed the lowest initial level of the six (8·7 units).

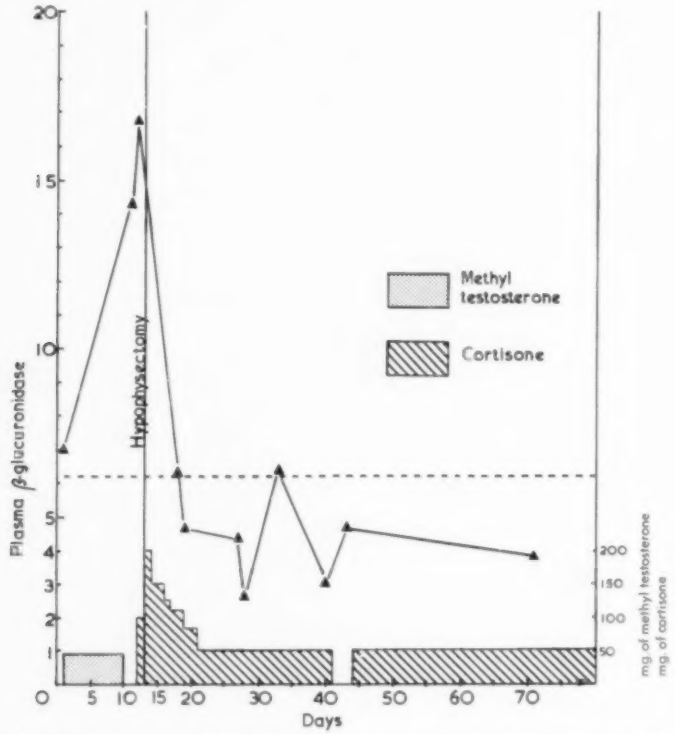

FIG. 1.—The effect of oral methyl testosterone on the plasma β-glucuronidase (Case 91). The dotted line represents the upper limit of normality.

On withdrawal of testosterone a fall in β-glucuronidase of over five units occurred in four cases, of over two units in one, and a slight rise (0·7 units) in one.

(ii) Four cases receiving intramuscular testosterone propionate until admission all had normal β-glucuronidase readings. After stopping the drug a slight fall occurred in one and a rise in another. In the third case (No. 9) testosterone was continued until the day of operation.

Case 14 is of interest in that she was admitted, immediately after a course of methyl testosterone by mouth, with an initial reading of 9·05 units of β-glucuronidase. After stopping the testosterone the glucuronidase level began to fall and continued to do so, eventually reaching 2·60 units, even though the patient com-

menced a course of intramuscular testosterone propionate 200 mg. daily four days after admission (Fig. 2).

(iii) Two of the four cases admitted after a course of non-virilizing androgens had initial readings within normal limits, one was slightly raised, and the fourth was very high. Of the three for whom readings are available after withdrawal of the hormone none showed any appreciable change.

In all, therefore, ten cases had oral methyl testosterone, and all ten had abnormally high β-glucuronidase levels while taking the drug or immediately after it had been discontinued. Eight of the ten showed a fall in β-glucuronidase subsequent to withdrawal of testosterone.

Of the 9 cases given intramuscular testosterone propionate or non-virilizing androgens only one case showed marked elevation of the β-glucuronidase, and this did not fall when the androstalone was stopped.

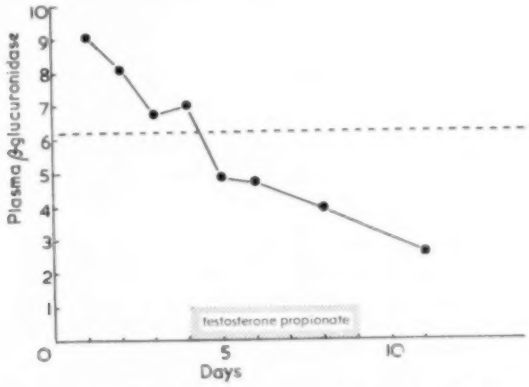

FIG. 2.—Case 14. High β-glucuronidase on admission after a course of methyl testosterone by mouth as an out-patient. On withdrawal of the methyl testosterone the β-glucuronidase fell, and this fall was not checked by the administration of intramuscular testosterone propionate. The dotted line represents the upper limit of normality.

(2) *The effect of ethinyl oestradiol and stilboestrol*

Nine cases received ethinyl oestradiol or stilboestrol. Six of these were under observation before the start of oestrogen therapy. They were each given ethinyl oestradiol in large doses by mouth for periods varying from four to twelve days, after which the drug was discontinued.

The pre-oestrogen mean β-glucuronidase for each case is given in Column 2, Table III. The mean β-glucuronidase values for the three days following, but not including the day on which the oestrogens were stopped, are given in Column 5, Table III, for the four cases in whom such measurements were possible.

The maximum levels reached after the end of the course, and before any subsequent fall in β-glucuronidase, are given in Column 7, Table III.

In all six cases a rise of β-glucuronidase was observed. This was marked in four and moderate in two. Three other cases had received stilboestrol until the time of admission (Table IV).

TABLE IV.—*Stilboestrol Until Time of Admission*

Case number	Initial β-glucuronidase	Interval since stilboestrol	Last β-glucuronidase before other treatment	Interval	Dose and duration	Subsequent rise or fall	Interval
70	26·00	1 day	22·60	3 days	2 months	Fall to 6·60 hypophysectomy intervening	34 days
98	10·10	1 day	7·42	7 days	7 weeks 10 mg. t.d.s.	Fall to 1·67 hypophysectomy intervening	31 days
109	10·50	1 day	9·05	7 days	10 weeks 5 mg. t.d.s.	Fall to 7·81, died 13 days after admission	8 days

TABLE V.—*Cortisone Administration. Failed Hypophysectomies*

Case number	Pre-cortisone mean β-glucuronidase	Number of estimations	Mean β-glucuronidase 1st week post-cortisone	Number of estimations	2nd week	Number of estimations	3rd week	Number of estimations	Type and dose of steroid	Operation	Androgens
9	3·55	1	4·62	3	—	—	—	—	Cortisone 25 mg. b.d. from 23rd post-op. day	Stereotaxic ^{198}Au.	Test. prop. I.M. + thiotepa
13	4·20	1	—	—	—	—	3·63	1	Cortisone 25 mg. b.d. from 19th post-op. day	Steteotaxic ^{198}Au.	Methyl test. until 14 days pre-op.
25	18·62	4	16·60	4	13·10	4	13·95	4	Prednisone 40 mg./day for 21 days from 7 days pre-op.	Steteotaxic ^{198}Au.	None
30	1·9	3	3·28	2	3·15	1	—	—	Routine cortisone cover	Open op. pre-fixed chiasm	None
106	11·27	6	12·97	3	(6·91)	3	(8·37)	2	Routine cortisone cover	Open op. severe bleeding	Androstalone until 14 days pre-op.
					patient given saccharo 1 : 4 lactone in 2nd and 3rd weeks						
107	6·67	4	7·98	4	5·54	3	—	—	Routine cortisone cover	Open op. severe bleeding	Durabolin until 6 days pre-op.

All three cases had an initially high β-glucuronidase. After withdrawal of the drug two showed a marked fall in the enzyme level. The third showed a slight fall but died as a result of a cerebral vascular accident after only thirteen days.

3. *The effect of cortisone*

In six cases pituitary ablation was attempted, but for various reasons not achieved. In one case severe bleeding from the fossa prevented removal of the pituitary, and in another a pre-fixed chiasm was present. In both cases ^{198}Au seeds were injected into the gland after it had been broken up as far as possible with a sharp probe. The third case was abandoned because of uncontrollable intracranial haemorrhage, and the fossa was not opened. The remaining three cases had ^{198}Au seeds implanted into the pituitary fossa by a stereotaxic technique (Bennett, 1950).

In the case with a pre-fixed chiasm (No. 30) thyroid function became slightly depressed post-operatively. However she continued to menstruate and the cortisone withdrawal test (Baron and Gurling, 1960) suggested that pituitary destruction was incomplete. In the remaining five cases no evidence of diminished pituitary function was obtained, and in all six cases the progress of the disease was unaffected.

The three cases subjected to open operation were given cortisone 100 mg. intramuscularly on the day before operation, 175–200 mg. intramuscularly and intravenously on the day of operation, and thereafter steadily decreasing doses until a maintenance dose of 25 mg. b.d. was reached on the tenth post-operative day.

Two of the cases submitted to stereotaxis did not start cortisone until nineteen and twenty-three days postoperatively. The third received prednisone 40 mg. daily for seven days before and for fourteen days after operation.

Two cases (No. 106 and 107) had received non-virilizing androgens until their admission. Case 13 had had methyl testosterone by mouth until admission, and the pre-cortisone level for this case is therefore taken as the single estimation immediately before operation and fourteen days after admission. Case 9 had received intramuscular testosterone propionate and also thiotepa until just before the gold implantation, and therefore the last single estimation before operation is taken as the pre-cortisone level of β-glucuronidase. Cases 30 and 25 had had no previous treatment.

In each case subsequent estimations of β-glucuronidase have been grouped according to the number of weeks elapsing since the start of cortisone therapy. The mean figures for each week are given in Table V.

Each of the three cases submitted to open operation showed a slight rise in β-glucuronidase in the first post-operative week, followed in later weeks by a fall towards the pre-operative level. The fall in enzyme level noted in the second and third post-operative weeks for Case 30 may be due to the fact that she received treatment with saccharo-1 : 4-lactone, a glucuronidase inhibitor.

In the two cases treated by stereotaxic implantation who did not start cortisone until some weeks after operation, a post-operative rise of β-glucuronidase was also observed (not shown in Table V). Estimations made after the start of cortisone therapy, however, do not differ greatly from the pre-operative figures.

Case 25 showed a moderate fall in β-glucuronidase, coincident with the start of prednisone. When this treatment was stopped the enzyme returned to its

former level. None of the five cases treated with cortisone shows any comparable alteration in enzyme level.

(4) *The relationship of pre-operative β-glucuronidase and response to hypophysectomy*

Pituitary ablation was performed in twenty-eight patients, twenty-one by open operation and seven by stereotaxic implantation of ^{90}Y rods into the fossa.

The results of these operations are classified into four groups :

I. *Major regression.*—Objective and subjective improvement maintained for more than two months.

II. *Minor regression.*—Subjective improvement maintained for more than two months or apparent cessation in spread of a formerly advancing lesion.

III. *Doubtful group.*—Equivocal subjective or objective improvement, or improvement lasting less than two months.

IV. *Failed group.*—Those cases in whom no objective or subjective improvement was obtained, and those dying within one month of operation.

Groups I and II combined are approximately equivalent to the " remission " group of Luft, Olivecrona and Ikkos (1958).

In view of the findings in respect of the effect of androgen and oestrogen administration on β-glucuronidase a direct comparison between the uncorrected pre-operative mean β-glucuronidase levels would obviously be misleading. An attempt has been made to correct for the effect of androgens by calculating the mean β–glucuronidase, either from estimations made before the administration of androgens, where this is possible, or from estimations made over a month after the cessation of androgen therapy. In a number of cases the hypophysectomy was performed within a month of the cessation of androgens, and these have been excluded from the calculation.

Two cases (No. 70 and 98) had received oestrogens until just before admission, and a further two (No. 55 and 104) had already undergone ablative endocrine surgery in the form of oophorectomy and adrenalectomy respectively, to which they had responded. These cases are also excluded.

When these corrections have been made nine cases are left in the combined I and II groups and ten cases in the combined III and IV groups (Table VI).

The mean β-glucuronidase level for the combined groups I and II is 5·74 units, while that for the combined groups III and IV is 9·22 units. This difference is statistically significant ($t = 2{\cdot}70$, $P = <0{\cdot}02$). If still more rigorous selection is carried out by excluding Cases 99, 102 and 96 who received prednisone until the time of their admission, Case 94 who survived only four days after implant of ^{90}Y and Case 91 whose pre-operative ^{131}I uptake was in the thyrotoxic range the numbers in groups I and II are reduced to eight and in groups III and IV to six. The mean values are 5·58 and 10·61 respectively, and the difference is still significant ($t = 3{\cdot}63$, $P = <0{\cdot}01$).

These figures are represented, graphically, together with subsequent changes in β-glucuronidase levels in Fig. 3 and 4.

(5) *The effect of hypophysectomy on plasma β-glucuronidase*

In order to present the available figures in a comparable form the post-operative estimations for each hypophysectomy patient are given as mean values for each post-operative week. Table VII is calculated from a total of 292 pre- and post-

operative figures. Those marked with an asterisk are those who were excluded from the calculation in Section 4 above, because of recent androgen or oestrogen administration. The figures in brackets represent the number of estimations from which each mean value has been calculated. Fig. 3 and 4 are a graphic representation of Table VII after exclusion of the cases marked with an asterisk.

It will be observed firstly that in all group I and II cases save one (No. 14) there is either no great alteration in level or else a downward trend of the β-glucuronidase (Fig. 3). Secondly only two cases show elevation above 7·5 units at any point.

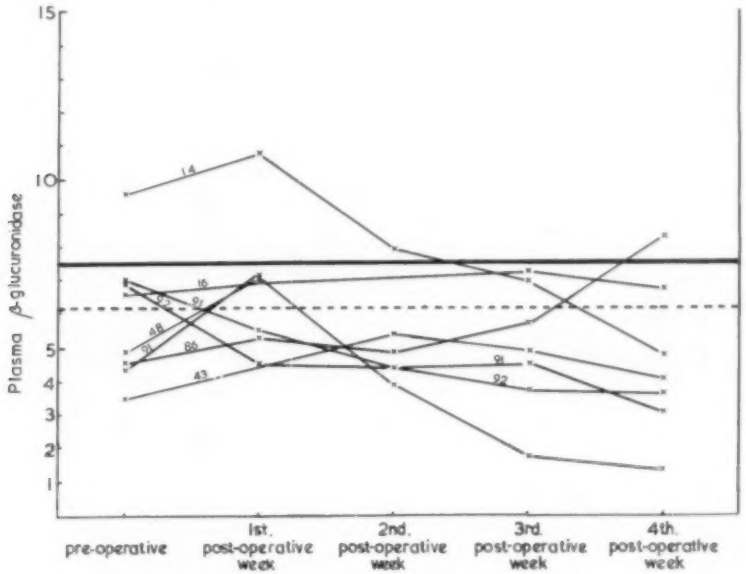

FIG. 3.—Cases responding favourably to hypophysectomy. Serial β-glucuronidase estimations before and after operation. The dotted line represents the upper limit of normality, the heavy continuous line the normal mean β-glucuronidase plus three standard deviations.

In groups III and IV however the variations from week to week are much more pronounced, there is no general tendency up or down and all save two are greater than 7·5 units (mean normal value plus three standard deviations), at some point (Fig. 4).

(6) *The effect of advance in the disease process on the plasma β-glucuronidase*

Twelve cases were followed for periods of over three months. The first available figure for each case is shown in Column 3, Table VIII. Wherever possible these figures refer to a period at least one month after cessation of androgen or oestrogen therapy, but in four of the cases this does not apply (No. 23, 7, 9 and 96). Each of these had received androgen therapy within one month of the initial reading quoted.

TABLE VI.—*Relation of pre-operative β-glucuronidase to Response to Hypophysectomy*

Case number	Pre-op. mean β-glucuronidase	Number of estimations	Pre-op. mean β-glucuronidase from 1 month after cessation of androgens	Number of estimations	Lesions and symptoms	Type of improvement	Duration of remission or survival
Group I :							
86	4·57	3	4·57	3	Large primary	Decrease in size	6 months +
					Pleural effusion	,,	
					Lungs	,,	
16*	6·59	7	6·60	2	Bone	Decrease in size	10 months
					Skin	,,	
					Lungs	,,	
					Bone pain	Less	
14*	9·56	4	9·56		Skin	Decrease in aize	5 months +
					Pain in breast	Abolished	
23	3·67	4	—	—	Bone	No change	4½ months
					Skin	Healed	
					Pleura	Decrease in size	
					Lungs	,,	
					Bone pain	Abolished	
48	4·84	3	4·84	3	Bone	Recalcified	13 months +
					Bone pain	Abolished	
70*	23·40	3	—	—	Bone	Recalcified	5 months +
					Skin	Healed	
					Pleura	,,	
					Bone pain	Abolished	
88*	4·54	4	—	—	Primary lesion	Diminished in size	9 months +
					Cerebral	Symptoms abolished	
					Raised intra-cranial pressure	Lowered	
89	8·07	5	4·33	1	Bone	Recalcified	8 months +
					Skin	Healed	
					Bone pain	Abolished	
91	12·68	3	7·00	1	Primary lesion	Dimunution in size	8 months +
					Bone	No change	
					Pleural effusion	Diminution in size	
87	4·36	4	4·36	4	Primary lesion	Diminution in size	8 months +
					Bone	No change	
					Bone pain	Abolished	
43	3·50	2	3·50	2	Bone	Marked regression	15 months +
					Bone pain	Abolished	
Group II :							
92	6·94	4	6·94	4	Bone	Not known	9 months +
					Bone pain	Abolished	
Group III :							
2	15·65	2	15·65	2	Liver	No change	3 months
					Skin	,,	
					Jaundice	Transient improvement	
7	9·08	5	—	—	Local recurrence	No change	5 months
					Glands	Transient dimunution	
					Skin	No change	

39	15·33	14	13·99	1	Bone	No change	40 days	
					Jaundice	Transient improvement		
					Ascites	No change		
					Bone pain	Transient improvement		
93	9·99	2	9·99	2	Mediastinum	No change	8 months +	
					Bone	,,		
					Recurrent laryngeal palsy	,,		
					Bone pain	Transient improvement		
Group IV:								
55	4·72	7	—	—	Liver	No change	8 months +	
					Ascites	,,		
81	18·80	4	—	—	Bone	No change	2 months	
					Glands	,,		
					Paraplegia	,,		
					Bone pain	,,		
94*	6·23	4	6·23	4	Primary lesion	No change	4 days	
					Glands	,,		
					Pleura	,,		
					Bone	,,		
95*	8·91	7	8·91	7	Primary lesion	No change	9 days	
					Liver	,,		
					Lungs	,,		
					Pleura	,,		
96*	10·24	6	10·24	6	Bone	No change	17 days	
					Pleura	,,		
					Bone pain	,,		
97	7·40	6	7·40	6	Bone	No change	18 days	
					Liver	,,		
					Peritoneum	,,		
98	8·76	2	8·76	2	Primary lesion	No change	7 months +	
					Pleura	,,		
99	8·02	7	8·02	7	Primary lesion	No change	1 month	
					Bone	,,		
					Bone pain	,,		
100	9·44	6	8·80		Primary lesion	No change	6 weeks	
					Bone	,,		
					Lungs	,,		
					Liver	,,		
					Brain	,,		
					Bone pain	,,		
102	4·09	3	4·09	3	Local recurrence	No change	5 months +	
					Mediastinum	,,		
					Internal mammary	,,		
103	6·49	8	—	—	Local recurrence	No change	6 months +	
					Bone	,,		
104	5·67	15	—	—	Local recurrence	No change	4 months +	
					Bone	,,		
					Glands	,,		
					Bone pain	,,		

* The cases marked with an asterisk were treated by stereotaxic implantation of 90*y* into the pituitary fossa. The remainder underwent open hypophysectomy.

At the time of their last available estimations five cases were in remission or static, and the mean net rise for these five cases is 0·75 units over eight months. All but one of the five final readings were within the normal range.

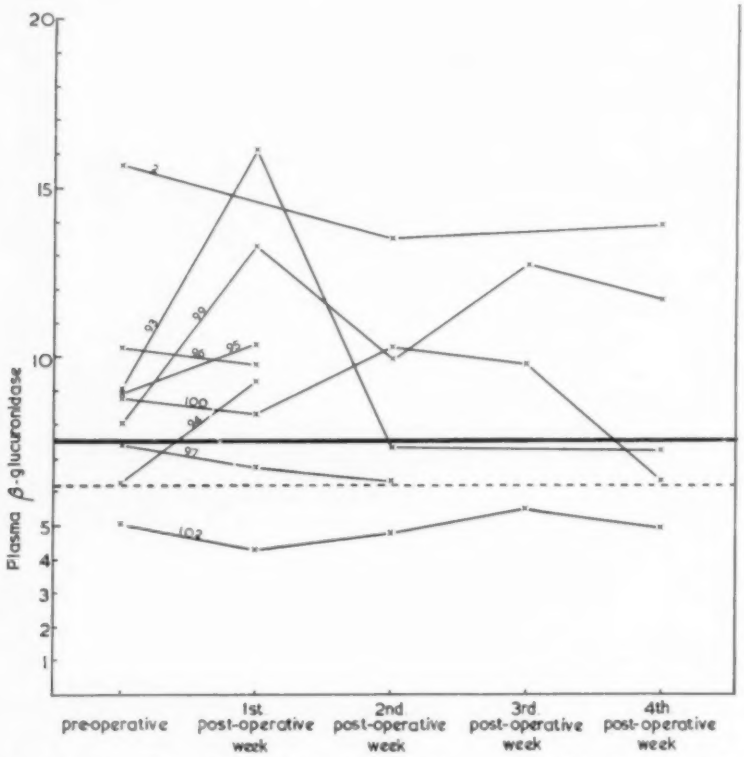

FIG. 4.—Cases responding unfavourably to hypophysectomy. Serial β-glucuronidase estimations before and after operation. The dotted line represents the upper limit of normality, the heavy continuous line the normal mean β-glucuronidase plus three standard deviations.

The remaining seven cases showed signs of advancing disease at the time of the final reading and the mean net rise for this group is 3·98 units over 5·8 months. The final readings of all but one of the seven were outside the normal range.

DISCUSSION

Cohen (1951) gave testosterone to six patients, but noted a rise in serum β-glucuronidase in only one. Fishman (1951) found that methylandrostenediol produced a rise in the serum enzyme level in a proportion of cases.

In this series administration of testosterone by mouth was fairly consistently associated with a rise in plasma β-glucuronidase, whereas testosterone propionate

TABLE VII.—*The Effect of Hypophysectomy on Plasma β-glucuronidase*

Case Number	Pre-op. β-glucuronidase	1st post-op. week	2nd week	3rd week	4th week	Response to hypophysectomy
14	9·56 (4)	10·79 (3)	7·96 (1)	7·00 (2)	4·80 (2)	Group I
16	6·60 (2)	7·00 (1)	—	7·25 (2)	6·79 (3)	,,
23*	3·67 (4)	4·72 (2)	5·32 (2)	4·68 (3)	3·11 (2)	,,
48	4·84 (3)	7·02 (3)	—	—	—	,,
70*	23·40 (3)	18·05 (2)	12·09 (3)	14·70 (1)	9·50 (2)	,,
86	4·57 (3)	5·28 (4)	4·82 (2)	5·67 (2)	8·30 (3)	,,
87	4·36 (4)	7·15 (1)	3·85 (2)	1·79 (2)	1·35 (1)	,,
88*	4·54 (4)	5·53 (1)	3·78 (1)	1·60 (2)	—	,,
89*	4·33 (1)	6·55 (3)	9·04 (1)	4·60 (3)	3·67 (3)	,,
91	7·00 (1)	5·56 (2)	4·40 (1)	4·52 (2)	3·02 (1)	,,
43	3·50 (2)	—	5·40 (1)	4·83 (2)	4·11 (2)	Group II
92	6·94 (4)	4·50 (1)	4·40 (2)	3·75 (1)	3·65 (3)	,,
2	15·65 (2)	—	13·50 (1)	—	13·90 (1)	Group III
7*	9·08 (5)	6·93 (2)	6·63 (4)	5·54 (1)	4·32 (4)	,,
39*	13·90 (1)	20·70 (4) (androgens intervening)	15·68 (3)	14·70 (2)	19·45 (2)	,,
93	9·00 (2)	16·12 (1)	7·31 (2)	—	7·24 (1)	,,
55*	4·72 (9)	—	10·90 (1)	4·51 (3)	3·13 (2)	Group IV
81*	18·80 (4)	8·02 (4)	4·05 (1)	6·11 (3)	7·93 (4)	,,
94	6·23 (4)	9·25 (1)	—	—	—	,,
95	8·91 (7)	10·39 (2)	—	—	—	,,
96	10·24 (6)	9·80 (1)	—	—	—	,,
97	7·40 (6)	6·66 (4)	6·34 (1)	—	—	,,
98*	8·76 (2)	6·19 (2)	2·53 (2)	2·11 (1)	14·06 (3)	Group IV
99	8·02 (7)	13·23 (1)	9·95 (2)	12·72 (3)	11·61 (1)	,,
100	8·80 (1)	8·35 (3)	10·30 (2)	9·80 (1)	6·35 (1)	,,
102	5·02 (4)	4·28 (3)	4·79 (2)	5·46 (3)	4·86 (1)	,,
103*	6·49 (8)	5·52 (2)	3·87 (2)	—	—	,,
109*	5·67 (15)	11·20 (2)	7·12 (2)	—	—	,,

* Cases excluded from the calculation in Section 4 of Methods.

The figures in brackets represent the number of estimations from which each mean value has been calculated.

TABLE VIII.—*Relation of Plasma β-glucuronidase to Deterioration in Clinical State*

Case Number	Period of follow-up (months)	First available reading	Last available reading	Rise or fall	Response to hypophysectomy	State at time of last available reading
7	14	3·94 (Test. prop.)	7·02	+3·08	Group III	Deteriorating
9	4	3·92 (Test. prop.)	7·10	+3·18	Pituitary not destroyed	Deteriorating
13	5	4·20	8·65	+4·45	Pituitary not destroyed	Deteriorating
16	10	6·60	5·20	−1·4	Group I	In remission
36	9	3·07	3·45	+0·38	Pituitary not destroyed	Static
23	6	3·67 (Androstalone)	5·4	+1·73	Group I	Deteriorating
30	6	1·9	4·10	+2·20	Pituitary not destroyed	Static
12	3	2·29 (post-hypophysectomy)	8·10	+5·81	Group IV	Deteriorating
43	8	2·96	6·85	+3·89	Group II	Static
48	7	4·84	3·56	−1·28	Group I	In remission
96	6	3·25 (Methyl test.)	9·80	+6·55	Group IV	Deteriorating
100	3	8·80	11·90	+3·10	Group IV	Deteriorating

administered intramuscularly did not produce a similar rise. Possibly this difference is due to a difference in conjugation of the testosterone according to its route of absorption.

No constant effect was demonstrated as a result of administration of non-virilizing androgens.

Other workers have frequently observed a rise in blood glucuronidase following administration of both natural and synthetic oestrogenic substances (Cohen, 1951 ; Goldbarg, Pineda, Banks and Rutenburg, 1959 ; Fishman, 1951 ; Cohen and Huseby, 1951) and these findings are confirmed by the present investigation. The fact that stilboestrol produces a similar effect on plasma glucuronidase to that of naturally occurring oestrogens, in spite of the lack of any chemical similarity, may be due to the metabolism of stilboestrol via its monoglucuronide.

Cohen (1951) reported that cortisone produced a rise in serum β-glucuronidase levels in man, but in this series no constant effect has been observed, either in the control group of six " sham-operated " cases or in those whose pituitaries were destroyed, though one case (No. 25) showed a fairly marked fall on prednisone therapy. A high proportion of cases showed a slight rise in β-glucuronidase in the first post-operative week, but this is probably a non-specific effect of trauma similar to the effect on urinary β-glucuronidase described by Boyland and Williams (1956) and Lewis and Plaice (1960).

A low plasma β-glucuronidase seems to be associated with a significantly greater chance of remission following hypophysectomy than is a high level. This is not an absolute criterion ; for instance Case 102 showed no response whatsoever to hypophysectomy yet had a pre-operative mean β-glucuronidase of 4·57. It may perhaps be significant that this patient had been taking prednisone until admission fourteen days before operation, and had shown a good response to hormone therapy in the past.

The percentage remission rate in the present series, after excluding those cases affected by recent androgen or oestrogen therapy is shown in Table IX.

TABLE IX

β-glucuronidase	Percentage remissions
0·96–6·20 (normal range)	83·5
6·20–7·50 (normal mean + 8 S.D. to mean + 3 S.D.)	60
7·50 +	12·5

These percentages are of course calculated from a relatively small sample and will probably require modification as more figures accumulate. Assuming however that they are fairly near the true figures the estimation of the plasma β-glucuronidase might well prove to be of value in deciding the form of therapy to adopt in a poor risk case. A figure of over 7·5 units, *in the absence of previous hormone therapy*, would be an indication for chemotherapy, rather than surgery.

The relationship of the plasma β-glucuronidase to the effect of hypophysectomy is probably indirect, certainly a fall in glucuronidase is not a necessary prerequisite of remission, nor does a fall in β-glucuronidase necessarily indicate that a remission is taking place.

In a number of patients the plasma β-glucuronidase was artificially lowered by oral administration of saccharo-1 : 4-lactone, a glucuronidase inhibitor, without any obvious change in the course of the disease. Probably the β-glucuronidase is merely an index of the relative proportions of the free and combined fractions of various steroids.

Finally, with regard to the relationship of the β-glucuronidase to advance in the disease process, Table VIII does suggest a direct relationship when the disease is followed over a sufficiently long period, a finding which is at variance with those of Cohen and Huseby (1951).

SUMMARY

Plasma β-glucuronidase levels of forty women and one man suffering from breast carcinoma have been studied.

(1) A rise in enzyme level was associated with administration of oral methyl testosterone, ethinyl oestradiol or stilboestrol.

(2) Intramuscular testosterone propionate, non-virilizing androgens and cortisone did not produce any constant effects on the enzyme.

(3) A comparison of the pre-operative β-glucuronidase levels of nineteen cases submitted to hypophysectomy showed a statistically significant difference between those who responded to the operation and those who did not. A low pre-operative level appeared to be associated with a high proportion of remissions.

(4) Follow-up cases over a prolonged period showed increasing enzyme titres with deterioration in the clinical condition.

(5) The significance of these results is discussed and it is suggested that the β-glucuronidase may prove to be a useful index of hormone sensitivity of breast carcinomas, providing the patient has not been receiving hormone therapy immediately before the estimation is made.

I should like to express my thanks to Mr. E. J. Radley-Smith for his encouragement of this project and for permission to study his cases. Also Dr. D. N. Baron and Dr. D. C. Williams for their guidance and expert advice on the technical and biochemical aspects of the investigation, Dr. Evelyn Boesen, Dr. Avery Denfield, Dr. Barbara Truscoe, Dr. Rosemary Khin Zaw and Sisters Hitchcock and Bennett for their unfailing co-operation in the documentation of the patients and collection of specimens.

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CELL-VIRUS INTERACTIONS WITH THE POLYOMA VIRUS: THE INDUCTION OF CELL TRANSFORMATION AND MALIGNANCY *IN VITRO*

D. MEDINA AND L. SACHS

From the Laboratories of Virology and Genetics, Weizmann Institute of Science, Rehovoth, Israel

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In the previous studies with polyoma virus reported in this series, an analysis has been presented of the cell-cirus interactions in tumour cells induced *in vivo* (Sachs and Winocour, 1959; Sachs and Fogel, 1960; Winocour and Sachs, 1961*a*), and of the lytic interaction after virus infection *in vitro* (Winocour and Sachs, 1960). In addition to the production and study of the lytic interaction *in vitro*, it is, of course, also desirable to produce and analyse *in vitro* the non-lytic interaction that results in the change of a normal into a tumour cell. In parallel with these studies, experiments were therefore undertaken on the development of a system for the *in vitro* induction of cell transformation and malignancy and on the use of this system for the investigation of cell-virus relationships. Data on the development of such an *in vitro* system for embryo and kidney cells from mice, hamsters and rats have previously been reported (Medina and Sachs, 1960; Sachs and Medina, 1961). There have also been independent reports of *in vitro* transformation after infection of mouse and hamster cells (Dawe and Law 1959; Vogt and Dulbecco, 1960). The present paper is concerned with the requirements for *in vitro* cell transformation including the use of virus mutants, and with the malignant nature and cell-virus relationships of the *in vitro* transformed cells. Transformation will be referred to in this paper as a stable non-lytic change in the cell produced as the result of virus infection.

MATERIALS AND METHODS

Culture media

The two media used in the present experiments consisted of 0·5 per cent lactalbumin hydrolyzate in Earle's saline, and Eagle's medium with a four-fold concentration of amino and vitamins. These media will be referred to as LA and EM, respectively. The media were supplemented with either horse serum (HoS) or calf serum (CaS) as indicated in the text.

Tissue culture

For the embryo tissue cultures, mouse embryos were excised at about the 15th day, rat embryos at about the 16th day, and hamster embryos at about the 11th day of gestation. Primary cultures were made with trypsin dispersed cell suspensions, prepared as described previously (Winocour and Sachs, 1960), at a seeding level of 8×10^6 cells with 10 ml. medium in 100 mm. petri dishes.

Secondary cultures were made after 4 to 5 days by seeding 2 to 2·8 $\times$ 10^6 cells (depending on the experiment) with 4 ml. of medium in 50 mm. petri dishes. Unless otherwise stated, mouse and rat cultures were prepared in LA and 10 per cent HoS. However since LA with either HoS or CaS, or EM with HoS, did not give sufficiently good monolayers of hamster cells, hamster primary and secondary cultures were made in EM and 10 per cent CaS. Plastic petri dishes (Falcon Plastic Co., U.S.A.) were generally preferred to glass petri dishes for the rat and hamster cells, since they gave better cell growth.

Kidney cultures were seeded at 2 $\times$ 10^6 cells in 50 mm. petri dishes. Those from young rats and hamsters were made with kidneys from 3 to 9 day old animals, and those from adults with kidneys from 2 to 3 months old animals. Hamster tumours induced *in vivo* were made into cell suspension as described previously (Sachs and Fogel, 1960), and seeded at 2 $\times$ 10^6 cells in 50 mm. petri dishes with EM and 10 per cent CaS.

All cultures were incubated in a humidified incubator supplied with a constant flow of 10 per cent CO_2 in air. In each experiment, the results were based on the comparison of at least 4 experimental and 4 control petri dishes.

Virus mutants

Three polyoma virus stocks originating from 3 sources have been used in these experiments, and virus from the 3 sources will be referred to as mutants IL11, BP5, and SP2. The IL11 virus, which could also be referred to as the wild type (Winocour and Sachs, 1959) was a re-isolation from a mouse parotid tumour induced by virus from 3919 (Stewart, Eddy and Borgese, 1958) obtained through the courtesy of Dr. S. E. Stewart and Dr. B. E. Eddy; BP5 was a plaque purified stock of a polyoma virus isolated in Toronto from a mouse mammary tumour, and kidney supplied by Dr. M. Stoker (Stoker, 1960); and SP2 was a small plaque mutant isolated after *in vitro* infection of L cells with a non-plaque purified stock of IL11 (Winocour and Sachs, 1961*b*). All virus stocks were made on mouse embryo monolayers, as for previous experiments (Winocour and Sachs, 1960, 1961*a*), from 3 times plaque purified virus.

Plaque assay, haemagglutination, and cell disruption for total virus

The plaque assay was carried out as described previously (Winocour and Sachs, 1960) using mouse embryo secondary monolayers with LA and 15 per cent HoS. The mouse embryos were in all cases taken from the inbred mouse strain C57B1/6. When glass petri dishes were used, an initial agar nutrient overlay of 8 ml., followed by an overlay of 2·5 ml. containing neutral red on day 6, was sufficient to maintain the monolayers for 21 days or longer (Winocour and Sachs, 1960). However, in order to maintain monolayers longer than 14 days on plastic petri dishes, it was found necessary to use an initial agar nutrient overlay of 10 ml., followed by 3 ml. of overlay containing neutral red on day 6.

Haemagglutination tests were made with guinea-pig red blood cells as described previously (Fogel and Sachs, 1959). To counteract any possible haemagglutination inhibitors, samples were heated at 56° C. for 30 min. before they were tested.

Cell disruption for total virus was carried out by removing the cells from the petri dishes with a rubber wedge, and vibrating the cell suspension at full power

for 3 minutes in a Raytheon 200-watt 10-kc magnetostrictive oscillator (Winocour and Sachs, 1960).

Animals

The mice used in the present experiments were from the inbred lines C57Bl/6, SWR, and DBA/2, whereas the rats (albino), and golden hamsters were from random bred colonies.

EXPERIMENTAL

Transformation of rat and hamster cells under different culture conditions

In order to determine optimum conditions for *in vitro* transformation of rat and hamster cells, the first experiments were concerned with the changes produced after *in vitro* infection, and subsequent growth of the cultures, in different media and sera, and at different incubation temperatures. This original series of experiments was carried out with virus BP5, and some of the results have already been reported (Sachs and Medina, 1961). Monolayers were infected with 0·1 ml. of virus suspension at a virus cell ratio of 3 to 10 plaque forming units (PFU) per cell and with an adsorption period of 3 hr. Both rat and hamster primary and secondary embryo cultures, and primary kidney cultures, were used.

In the experiments with different media, 5 and 10 per cent serum was first tested, and 10 per cent was the then chosen in all further experiments. Rat cultures were tested in LA or EM with HoS or CaS, but since LA with either HoS or CaS was not an adequate medium for the growth of hamster cells, hamster monolayers were made in EM and CaS and then tested in EM and HoS or CaS. The experiments on different temperatures originated from the results on the effect of temperature on the frequency of lysogenisation with bacteriophage (Luria *et al.*, 1958), and it had been found in some of the original experiments that a much clearer transformation with BP5 could be obtained in cultures maintained at 24° C. for 5 to 8 days after infection, and then transferred back to 37° C., when compared to cultures kept at 37° C. only (Sachs and Medina, 1961). After growth of the monolayers at 37° C., most experimental groups therefore contained cultures maintained after infection either at 37° C. only, or at 24° C. for 5 to 8 days after infection.

The results of these experiments with BP5 are given in Table I. The data confirm those obtained previously (Sachs and Medina, 1961) in showing that *in vitro* transformation could be obtained in the appropriate conditions in all the tissues tested. The most suitable medium for transformation of each tissue, as established from these experiments, is also given in Table I. Secondary embryo cultures were more uniform in appearance than primaries, so that transformation could be more easily observed in secondaries. In the HE cultures, CaS tended to cause elongation of the cells after some days even in the controls. The use of EM and HoS after infection rather than EM and CaS, thus often produced a clearer early difference between the infected and control cultures. It should be mentioned that even with the most suitable medium, there was still occasionally some variation in the transformation observed, and the causes of this variation are being further studied. In all the cultures maintained at 24° C., there were only rarely slight changes at this temperature, and transformation was always obtained after the cultures were put back to 37° C. It should however be noted that transformation could also be obtained without the low temperature treatment.

TABLE I.—*Changes[a] Inducted by Polyoma Mutant BP5 After* in vitro *Infection of Rat and Hamster Cells*

Tissue culture	LA	Number of positive experiments[b]				The most suitable medium chosen[c]
		+ HoS	LA + CaS	EM + HoS	EM + CaS	
RE[d] primary	.	4/4[e]	1/1	1/4	1/5	LA + HoS
RE secondary	.	5/5[e]	3/3	0/1	NT	LA + HoS
RK young	.	1/1	NT	0/1	0/1	—
HE primary	.	NT[f]	NT	3/5	5/5	EM + HoS or CaS
He secondary	.	NT	NT	3/4[e]	1/2	EM + HoS or CaS
HK young	.	NT	NT	7/11[e]	3/4	EM + CaS
HK adult	.	NT	NT	4/6	2/2	EM + CaS

[a] Including morphological transformation of the cells, acidity, and other changes mentioned in the text.

[b] Most of the groups with more than 1 experiment were tested at 24° C. → 37° C. and at 37° C. only. All cultures were infected at a virus : cell ratio of 3 to 10 PFU per cell.

[c] 5 and 10 per cent serum were tested, and 10 per cent was then thosen for all further experiments.

[d] RE = rat embryo. RK = rat kidney. HE = hamster embryo. HK = hamster kidney.

[e] These groups showed a much clearer transformation when kept after infection for 5 to 8 days at 24° C. and then transferred to 37° C.

[f] LA was found to be an unsuitable medium for the growth of hamster cells.

The appearance of transformed rat and hamster cultures

As in the previous experiments (Sachs and Medina, 1961), several differences were noted between the infected and the control cultures. The changes included, in the infected cultures, changes in cell morphology; what is presumably a decrease of contact inhibition; increased acidity; better survival; and a difficulty in dispersing the cells with trypsin. Experiments are now in progress to determine to what extent all or only some of these *in vitro* changes are associated with transformation to neoplasia.

The main change in cell morphology in RE and HE was the elongation of cells (fusiform transformation, Fig. 1). The cells first become starlike, and then more elongated, and this morphological change was seen more clearly in cultures without a frequent medium change.

Another change which was particularly clear in HK and HE cultures, and was also seen in the rat cultures (Fig 1), was a criss-cross type of growth, which will be referred to as a decrease of contact inhibition (Abercrombie, Heaysman and Karthawser, 1957).

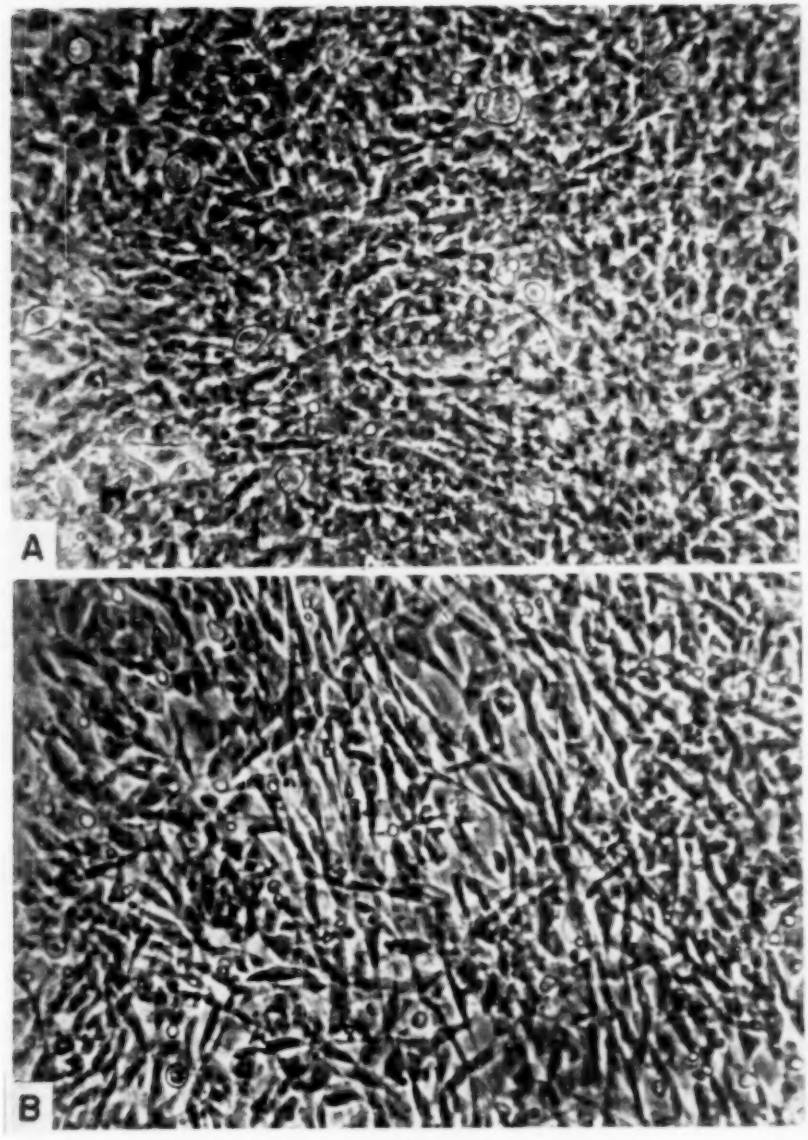
A very common change in all cultures was a marked increase in acidity, so that the medium of the infected cultures was more acid than in the controls. After changing the medium, it could be shown that the transformed cultures showed an increased acidity in less than 24 hr. after the medium change, whereas the number of cells in the transformed and control cultures was the same.

EXPLANATION OF PLATE

FIG. 1.—Living rat embryo secondary cultures.

A = Control cultures.

B = Fusiform shape of cells and criss-cross growth at 12 days after *in vitro* infection with SP2 at a virus : cell ratio of 5 PFU per cell. Phase contrast. ×170.



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A better survival of infected cultures was found in both RE and HE by keeping cultures sufficiently long without medium change, or under agar. Whereas the cells of control cultures degenerated, under these conditions, there was no such degeneration in the infected cultures. When old cultures started to peel off the plate, a much faster shrinkage was noted in the infected (and especially in the transformed) cultures, which formed a compact ball of tissue in contrast to the much looser shrinkage of the controls. It was also found that whereas control cultures could be readily trypsinised and formed cell suspensions in the usual way, transformed cultures treated with the same trypsin solution came off the glass with difficulty and did not form good cell suspensions.

RE monolayers infected with 1×10^7 PFU of the mutant SP2 (see next section) previously incubated with rabbit anti-polyoma antiserum for 30 min. showed no *in vitro* transformation.

There was no obvious cytopathic effect in any of the experiments shown in Table I, although a cytopathic effect was found after virus infection of HE with SP2. Some cell deaths seemed to occur in infected RE cultures, but the appearance of the spherical cells, free in the medium, was not the same as the clusters produced after the cytopathic effect in mouse and hamster cultures. Infected RE monolayers also often showed groups of small cells, or what may have been cell fragments, not found in the controls.

Transformation with different virus mutants

In a search for mutants with a better *in vitro* transforming activity than BP5, tests for transformation were made with the two other polyoma mutants IL11 and SP2. Of these 3 mutants, BP5 and IL11 produce large plaques and have a PFU : HA ratio of 1×10^5 (Winocour and Sachs, 1960), whereas SP2 produces small plaques and has a PFU : HA ratio of 1×10^6 (Table II). The

TABLE II.—*Plaque Size and Haemagglutination of 3 Polyoma Mutants*[a]

Virus mutant	Virus origin[b]	Plaque size	PFU : HA ratio
SP2	*In vitro* infected L cells	Small	1×10^6
IL11	Mouse parotid tumour	Large	1×10^5
BP5	Mouse mammary tumour	Large	1×10^5

[a] The virus stocks compared in this table all had titers of 1×10^8 PFU per ml.
[b] For further details see Materials and Methods.

plaques of BP5 and IL11 when tested on mouse embryo monolayers in LA and 15 per cent HoS usually appear on day 7 or 8 and are about 12 mm. in diameter on day 20. Plaques of SP2, like those of SP1 (Medina and Sachs, 1960), usually appear 2 to 3 days later i.e. on day 10 or 11, show almost no increase in size, and are about 0·5 mm. in diameter on day 20. Neutralisation tests have shown that there is cross neutralisation between SP1, SP2, BP5 and IL11.

The comparative transformation tests with BP5, IL11, and SP2 were made with RE and HE secondaries inoculated at a virus : cell ratio of 3 to 10 PFU per cell. After the production of monolayers at 37° C., the cultures were infected and then grown either at 24° C. for 7 days and then transferred back to 37° C., or at 33° C. only, or at 37° C. only. The RE cultures were tested in LA and 10 per cent HoS, and the HE cultures in EM and 10 per cent HoS. In each experi-

ment the 3 mutants were tested together on monolayers made from the same embryos prepared at the same time. The earliest appearance of morphological transformation was recorded, and the results are given in Table III. These data show that there was a difference in the earliest appearance of morphological transformation produced by the 3 mutants.

TABLE III.—*The Earliest Appearance of Transformation With Different Virus Mutants*

Virus mutant	24° → 37°[a] HE[b]	33° RE	33° HE	37° RE	37° HE	Average RE	Average HE
SP2	3[c]	2	2–4	2	3	2	3
IL11	3	4	9	4	4	4	5–6
BP5	3	7	3–13	7	13	7	7–8

[a] Seven days at 24° after infection and then transferred to 37° C.
[b] HE = hamster embryo. RE = rat embryo.
[c] Day when transformation was first observed, not including days at 24° C. All cultures were infected at a virus : cell ratio of 3 to 10 PFU per cell.

It can be seen from these results, that in the 24° C. → 37° C. experiments all 3 mutants showed the earliest appearance of transformation 3 days after returning the cultures to 37° C. But in cultures kept at either 33° C. or 37° C, only, SP2 produced the earliest and BP5 the latest transformation. The mutants can thus be arranged according to the speed of transformation in mass cultures in the order SP2-IL11-BP5. The experiments with BP5 again show a generally earlier appearance of transformation in 24° C. → 37° C. than in 37° C. or 33° C. only, and the same also seems to apply to IL11. It is significant that an obvious transformation can be observed with SP2 as early as 2 days after infection, thus indicating that cell transformation by polyoma virus can be a rapid event that can occur soon after virus infection.

The minimum virus inoculum required for transformation of rat and hamster cultures

During the course of attempts to obtain discrete foci of transformed cells for quantitative studies and for the comparison of plaque forming units with transforming units, it was noted that there seemed to be a minimum virus inoculum below which no transformation was observed after infection of undisturbed cultures, i.e. cultures that had not been passaged after infection. Cultures containing $2{\cdot}5 \times 10^6$ RE or HE cells were therefore infected with different amounts of BP5 or SP2, in order to determine the time of transformation with different virus inputs and the minimum virus inoculum required for transformation. The results of such experiments at 24° C. → 37° C., and at 33° C., are given in Table IV.

The data show with both BP5 and SP2, that there was a considerable increase in time before transformation was observed as the virus : cell ratio of the inoculum decreased, and that no transformation was observed in any of the experiments with an input less than about 10^4 PFU per culture. Since experiments number 164 and 141 were observed for 20 and 24 days respectively, this time would appear to have been sufficiently long to detect any cell transformation in the monolayer

TABLE IV.—*The Minimum Virus inoculum for Transformation in Mass Cultures*

Experiment No.	Type of cell	Virus mutant	Temperature	Number PFU inoculated per plate[a]	Virus : cell ratio	Transformation first observed on day[b]	Duration of experiment (days)
164	HE[c]	BP5	24°→37°[d]	5×10^6, 5×10^5	2 : 1, 1 : 5	2	20
	HE	BP5	24°→37°	5×10^{4e}	1 : 50	13	
194	HE	SP2	33°	1×10^7	4 : 1	6	13
	HE	SP2	33°	1×10^6, 1×10^5	1 : 2·5, 1 : 25	10	
141	RE	BP5	24°→37°	1×10^7	4 : 1	1	24
	RE	BP5	24°→37°	1×10^6	1 : 2·5	8	
	RE	BP5	24°→37°	1×10^{4f}	1 : 250	12	
191	RE	SP2	33°	1×10^7	4 : 1	2	12
	RE	SP2	33°	1×10^6	1 : 2·5	10	

[a] Lower inoculations of virus, which did not transform the cultures, do not appear in this table.
[b] Not including days at 24° C.
[c] HE = hamster embryo. RE = rat embryo.
[d] Seven days at 24° C. and then transferred to 37° C.
[e] Inoculum of 5×10^3 PFU showed only acidity in the inoculated cultures.
[f] Inoculum of 1×10^5 PFU was not tested in this experiment.

especially in the RE cultures where morphological transformation was readily observed. These results thus indicate that a minimum virus inoculum is required for cell transformation to be observed in the undisturbed infected rat and hamster cultures.

Changes in tissues other than rat and hamster

In addition to the experiments with rats and hamsters, cells from rabbit embryo, human amnion, monkey kidney, and mouse embryo, were tested for *in vitro* transformation either with SP2 or with all 3 mutants. The results of these tests are given in Table V.

In 13 experimental groups with rabbit embryo secondary cultures which included the use of different temperatures, the 3 virus mutants, and EM with either CaS or HoS, the only change observed was an increased acidity of the virus infected cultures in one experiment. The possible effect of LA could not be tested with rabbits, since it was found to be an unsuitable medium for the adequate growth of rabbit cells. In experiments with SP2 and growth at 37° C. no change was found after infection of human amnion primaries, whereas an increased acidity was noted after infection of monkey kidney primaries.

Infection of mouse embryo cultures with SP2 produced a rapid fusiform transformation which was observed as early as 1 day after infection in an experiment at 33° C., and 2 days after infection in another experiment at 37° C. (Table V). The early appearance of this transformation confirms the results with rat and hamster cells in showing that cell transformation can be a rapid event that takes place soon after virus infection. Fusiform transformation has also been found after infection of mouse embryo cells with IL11 and BP5, and after infection of mouse kidney cells (Sachs and Medina, 1961).

TABLE V.—*Changes After* in vitro *Infection of Cells Other than Hamster and Rat*

Type of cell	Virus mutant	Medium	Temperature	Nature of change in infected culture	Changes first observed on day[c]	Duration of experiment (days)
Rabbit embryo	IL11	EM + HoS or CaS	24°→37°[a]	Acidity[b]	3	15
			33°	No change	—	15
			37°	„ „	—	15
	BP5	do.	24°→37°	„ „	—	15
			33°	„ „	—	20
			37°	„ „	—	15
	SP2	do.	33°	„ „	—	15
			37°	„ „	—	15
Human amnion	SP2	EM + HoS	33°	„ „	—	14
Monkey kidney	SP2	EM + CaS	33°	Acidity and swollen cells	5	14
Mouse embryo	SP2	EM + CaS	30°	Increased granulation	8	15
			33°	Fusiform transformation	1–3	11
			37°	„ „	2	6

[a] Seven days at 24° C. and then transferred to 37° C.
[b] This group was tested only in EM + CaS.
[c] Not including days at 24° C. All cultures were infected at a virus : cell ratio of 2 to 5 PFU per cell.

Tumour formation in vivo *by growth of* in vitro *transformed cells*

The experiments on *in vitro* cell transformation described above, have been combined, in the case of hamsters and mice, with tests for tumour formation *in vivo* by inoculating transformed cultures into adult animals. Adult animals were used in order to differentiate tumours produced by growth of transformed cells from those induced in the host by virus in the inoculum, and to avoid an early death of the host animals.

In the hamster experiments, including those reported earlier (Sachs and Medina, 1961), 66 animals, 1 to 8 months old, were inoculated with transformed or normal control hamster embryo cells. The cells were removed from the petri dishes by peeling off fragments of the cell layer with a rubber wedge, and each animal was injected subcutaneously in the middle of the back with 1 to 3 $\times$ 10^6 cells in EM and 10 per cent HoS. The results (Table VI) show that tumours were formed in 8/42 hamsters inoculated with cells from transformed cultures, but that there were no tumours in 24 hamsters inoculated with cells from non-infected control cultures.

All the tumours in these experiments grew progressively, and were localised at the site of injection of the cells. They grew either under the skin or intradermally, and histologically they were classified as sarcomas. The intradermal neoplasms were very hard, and in addition to amorphic material, they included cartilage and bone, that had apparently differentiated from the embryonic cells. In only one animal (the tumour palpable after 42 days), was a tumour found in addition to that at the site of inoculation, and this animal died with a heart

TABLE VI.—*Tumour Formation in Adult Hamsters by Growth of in vitro Transformed Hamster Embryo Cells*

Virus mutant	Temperature treatment of culture	Days after infection	Days after transformation	Number of animals with tumour / Number animals inoculated	Tumour first palpable (days after inoc.)	Observation period (months)
BP5	24°→37°[a]	12, 38, 38	1, 28, 28	3/22	48, 37, 54	5–6
	33°	17	14	0/4	—	5
SP2	24°→37°	12	1	1/4	42	5
	33°	15, 17[b]	11	4/7	52, 25, 129	5
	37°	15	15	0/5	—	4
Total	—	—	—	8/42	—	—
Controls (non-)	24°→37°	12, 38	—	0/16	—	5–6
	33°	15	—	0/5	—	4–5
	37°	15	—	0/3	—	4–5
Total	—	—	—	0/24	—	—

[a] Seven days at 24° C. and then transferred to 37° C.
[b] Tumours developed only in animals inoculated with transformed cells from the 15-day group.
[c] All cultures were infected at a virus : cell ratio of 5 PFU per cell.

tumour that may have been produced by virus in the inoculum. The time of appearance of palpable tumours in adult animals, the strict localisation of the tumour at the site of cell inoculation, and the existence of differentiated embryonic cells, all indicate that these tumours were produced by growth of transformed cells, and not induced in the host by virus in the inoculum. Even the 2 tumours that were first palpable at 129 days after cell inoculation into 8 months old hamsters, were strictly localised at the site of inoculation and there were no tumours at other sites in the animal. It is thus possible that these late tumours were also produced by growth of the *in vitro* transformed cells, and they have therefore been included in Table VI and VII.

Transplantability *in vivo* was tested in the case of the tumours palpable at 54, 52, and 25 days, and all three tumours gave progressively growing transplants after grafting into adult hamsters. The hard tumours became soft on the first passage.

It can be seen from Table VI that although tumours were produced after *in vitro* transformation with both BP5 and SP2, not all the inoculated transformed cultures resulted in the *in vivo* development of neoplasms. The *in vivo* growth of transformed cells did not seem to be influenced by the sex of the recipient, but the data in Table VII indicate, that the number of animals with neoplasms, and the time of appearance of palpable tumours, seemed to be influenced by the age of the host.

In tests with BP5 on tumour induction after subcutaneous inoculation of virus only into adult animals, no tumours were observed 3 months after inoculation of 1×10^7 PFU into 5 hamsters injected when 1 month old, or 6 months after inoculation of 3×10^7 PFU into 5 hamsters injected when 5 months old. In a test with IL11, subcutaneous tumours were found, in 2 out of 5 hamsters, 5 months after inoculation of 4×10^6 PFU into 2 months old animals. It is of

TABLE VII.—*The Incidence of* in vitro *Induced Hamster Tumours in Different age Groups*

Age of animals at time of inoculation (months)	Number of animals with tumours		Number of animals inoculated
	Early[a] appearing	Late[b] appearing	
1–1·5	6	0	31
4–6	0	0	7
8	0	2	4
Total	8[c]		42[d]

[a] Tumours palpable between 25 to 54 days after inoculation.
[b] Tumours palpable only at 129 days after inoculation.
[c] 3 ♂ and 5 ♀.
[d] 16 ♂ and 26 ♀.

interest to note that in this particular case, in contrast to the 2 tumours that were first palpable at 129 days after inoculation of *in vitro* transformed cells, and where the tumour bearing animals were still alive 4 months later, the 2 hamsters with tumours after inoculation of virus only died 2 to 3 weeks after the subcutaneous tumours were first palpable, presumably due to tumours at other sites.

In the experiments with mice, 101 animals, 1 to 6 months old, belonging to the inbred strain C57Bl/6, SWR, and DBA/2, were injected subcutaneously with 1×10^6 cells per animal of transformed or normal control isologous mouse embryo cultures. Although some of the cultures gave palpable growths *in vivo* these growths in all cases regressed, and no progressive growth of the cells was found in any of the mice (Table VIII).

TABLE VIII.—*The Absence of Tumour Formation by Growth of* in vitro *Transformed Mouse Embryo Cells Inoculated into Isologous Adult Mice*[a]

Mouse strain	Inoculated tissue	Temperature treatment of culture[b]	Inoculated days after infection	Number of animals inoculated	Progressively growing tumour	Observation period (months)
C57Bl/6	Transformed	33°	7	20	0	5
	Normal control	33°	7	8	0	5
	Transformed	37°	7	12	0	5
	Normal control	37°	7	4	0	5
SWR	Transformed	33°	7	12	0	5
	Normal control	33°	7	12	0	5
	Transformed	37°	7	20	0	5
	Normal control	37°	7	8	0	5
DBA/2	Transformed	33°	9	3	0	3
	Normal control	33°	9	2	0	3

[a] *In vitro* transformation by virus SP2.
[b] Most groups were grown both in LA and EM with HoS or CaS.
All cultures were infected at a virus : cell ratio of 5 PFU per cell.

It should however be noted, that it has been shown in all the 3 strains of mice used in these experiments (Sachs, 1961), that the transplantability of readily transplantable mouse tumours can be inhibited by inoculation of mice receiving the tumour grafts with polyoma virus. All the *in vitro* transformed cultures

contained virus, so that the absence of tumour formation *in vivo* by growth of the transformed mouse cells, does not necessarily mean that the transformed cells were not malignant. Further experiments to elucidate this point are now in progress.

Virus growth after infection of normal hamster cells

With the development of a system for the *in vitro* transformation of normal cells with polyoma virus, it was obviously of interest to determine the response of transformed cells to challenge infection. The results of challenge infection with tumour cells induced *in vivo* have previously been reported (Winocour and Sachs, 1961*a*), but the advantage of using *in vitro* transformed cells would lie in the theoretical possibility to test the response to challenge infection soon after cell transformation. As a control to such challenge experiments with transformed hamster and rat cells, a study was first made of the growth of polyoma virus after infection of normal cells.

Virus growth on normal hamster cells was determined with BP5 and SP2. Secondary HE cultures grown in EM and 10 per cent CaS, and containing 2×10^6 cells at the time of infection, were inoculated at 1 to 2 days after seeding with 0·1 ml. of stock virus at a virus : cell ratio of 5 PFU per cell. After 3 hours adsorption, the plates were washed 3 times, and 4 ml. of EM and 10 per cent HoS added. Free virus (FV) and/or total virus (TV) was assayed at various times in the same way as in previous experiments (Winocour and Sachs, 1960, 1961*a*) and virus yields were determined from a pool of 2 plates for each point on the growth curve. Additional $NaHCO_3$ was added to the medium when infected cultures became too acid.

A comparison between FV and TV in a growth curve for BP5, in cultures maintained at 24° C. for 7 days after infection and then transferred to 37° C., is given in Fig. 2. There was no growth of the virus at 24° C., but a sharp rise between the 8th and 9th day. The difference between TV and FV on day 9 indicates that as in the case of mouse embryo cells (Sachs, Fogel and Winocour, 1959 ; Winocour and Sachs, 1960) there is a considerable amount of cell associated virus in virus yielding hamster cells.

In another experiment with BP5, a comparison was made between growth at 24° C. → 37° C. and growth at 37° C. only, on HE secondaries prepared from the same embryos at the same time. The results for TV are given in Fig. 3. The curve for 24° C. → 37° C. again shows no growth at 24° C. and a sharp rise between the 8th and 9th day, whereas cultures maintained at 37° C. only show a much lower rise, and only a total increase from 8×10^3 PFU to $1{\cdot}5 \times 10^5$ PFU during a period of 15 days.

The results for TV at 37° C. and 33° C. only, after infection with SP2 are given in Fig. 4. In this experiment, a cytopathic effect, in addition to transformation, was found on day 4 at both temperatures. Although the rise at 33° C. seems to be somewhat later than the rise at 37° C., at both temperatures the final yield with SP2 was as high as that found after infection of mouse embryo cells (Winocour and Sachs, 1960).

These growth curves on HE thus indicate that after infection with BP5 the total yield per culture was higher in the experiment at 24° C. → 37° C. than at 37° C. only, and that at the latter temperature the virus yield was relatively low ; and furthermore that the highest virus yields were obtained after infection with

SP2 and growth at either 33° C. or 37° C. only. These results also suggest that there is a difference in the growth of BP5 and SP2, and this is now being further studied.

In order to determine the percentage of virus producing cells, HE cultures were infected with SP2 at a virus : cell ratio of 5 PFU per cell, with an adsorption period of 3 hr. The cultures were then washed 3 times and the cells suspended by trypsinisation. The cells were then again washed, incubated for 15 min. with antipolyoma antiserum, diluted, and cell dilutions plated for an infective

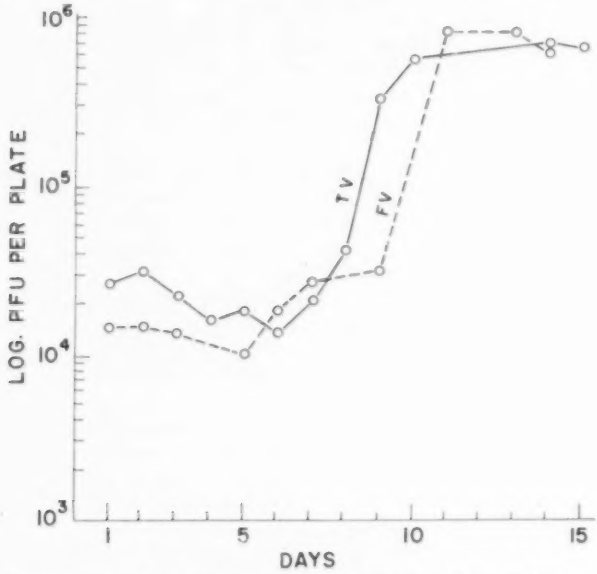

FIG. 2.—Growth curve of polyoma mutant BP5 on hamster embryo cultures infected at a virus : cell ratio of 5 PFU per cell. The cultures were maintained for 7 days at 24° C. after infection, and then returned to 37° C. The medium was changed on day 7.
TV = Total virus. FV = Free virus.

centre assay on mouse embryo monolayers. The hamster cells were fixed to the assay plate by placing a thin layer of nutrient agar on top of the cell suspension before adding the final amount of agar nutrient overlay (Winocur and Sachs, 1960). Plaques were counted on day 21 of the plaque assay, and the number of virus producing cells was determined by the relationship between the number of cells plated and the number of plaques. The HE cultures were grown at 37° C. only, and the percentage of virus producing cells was tested at time O, i.e. after the adsorption period, and at 7 days after infection.

The results gave 11 per cent virus producing HE cells at time O, and 4 per cent at day 7 (transformation in this experiment was observed on day 5). There was thus in this experiment no increase, and possibly even a decrease, in virus producing cells after transformation. The value of 11 per cent virus producing cells at time O, after infection with a virus : cell ratio of 5 PFU per cell, is com-

parable to the value obtained after infection of mouse embryo cells with IL11 (Winocour and Sachs, 1960).

Virus growth after infection of normal rat cells

Virus growth in cultures of normal rat cells was also determined with BP5 and SP2, and experiments were made at 24° C. → 37° C. and at 37° C. only. RE

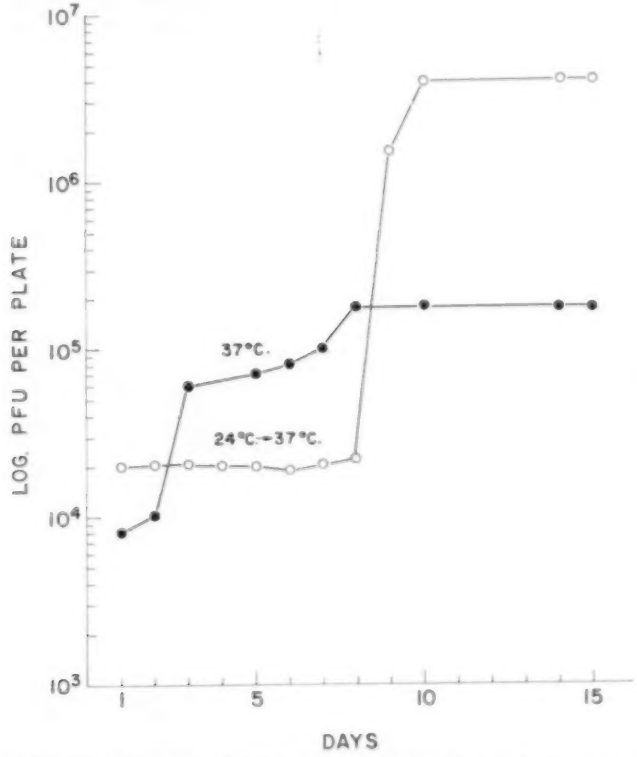

FIG. 3.—Growth curve (total virus) at 24° C. → 37° C., and at 37° C., of polyoma mutant BP5 on hamster embryo cultures infected at a virus : cell ratio of 5 PFU per cell. In the 24° C. → 37° C. curve the cultures were maintained for 7 days at 24° C. after infection and then returned to 37° C. Both curves were determined on cultures prepared from the same embryos at the same time. The medium was changed on day 7.

secondary cultures, taken at 1 to 2 days after seeding, were infected at a virus : cell ratio of 3 to 5 PFU per cell with an adsorption period of 3 hr. The cultures were then washed 3 times, 4 ml. of LA and 10 per cent HoS added, and TV content determined at various times. All the infected cultures in these experiments were transformed in the usual way. The results of 4 experiments, 2 with SP2 and 2 with BP5, each made with RE secondaries from a different batch of embryos, are given in Fig. 5. The data show that no virus growth was detected with either

BP5 or SP2 at 24° C. → 37° C. or at 37° C. only. There has also been no detectable virus growth after infection of RE with virus IL11 and growth at 37° C. (Sachs and Winocour, 1959; Sachs, 1961*a*).

The response of transformed cells to challenge infection

In order to determine the response of transformed cells to challenge infection, cultures of *in vitro* transformed cells, and of tumour cells induced *in vivo*, were challenged with polyoma virus and tested for TV content.

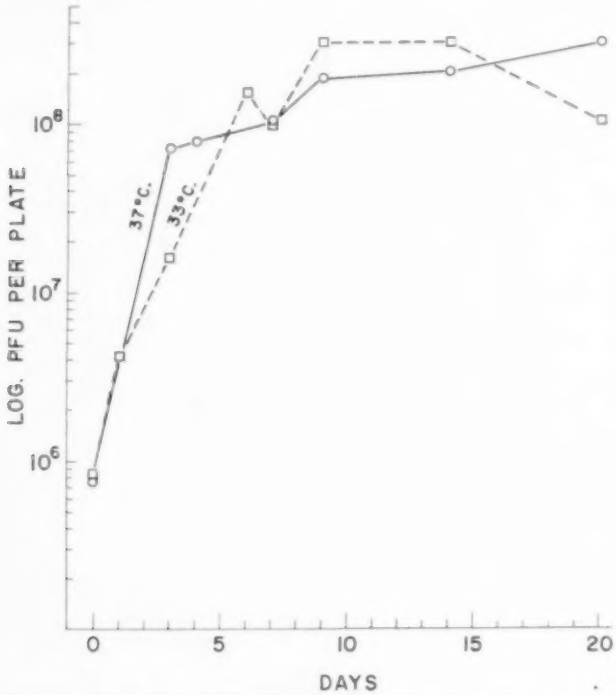

FIG. 4.—Growth curve (total virus) at 33° C., and at 37° C., of polyoma mutant SP2 on hamster embryo cultures infected at a virus : cell ratio of 5 PFU per cell. Both curves were determined on cultures prepared from the same embryos at the same time. The medium was changed on day 8 and day 13.

In the experiments with *in vitro* transformed HE cells, cultures were taken at 14, 19 and 24 days after infection (to ensure as complete a transformation as possible) and challenged, with their controls, with either BP5 or SP2 at a virus : cell ratio of 3 PFU per cell. The results of one such challenge experiment, after an original infection with SP2 and challenge with SP2 are shown in Fig. 6. In this experiment the cells were challenged 19 days after the original infection (15 days after the first appearance of transformation) and the cultures were kept for 10 days at 33° C. after the challenge inoculation. It has been shown (Fig 4),

that the final yield with SP2 at 33° C. was the same as at 37° C., and the use of 33° C. had the advantage in that the cultures could be readily maintained for 10 days even without a medium change.

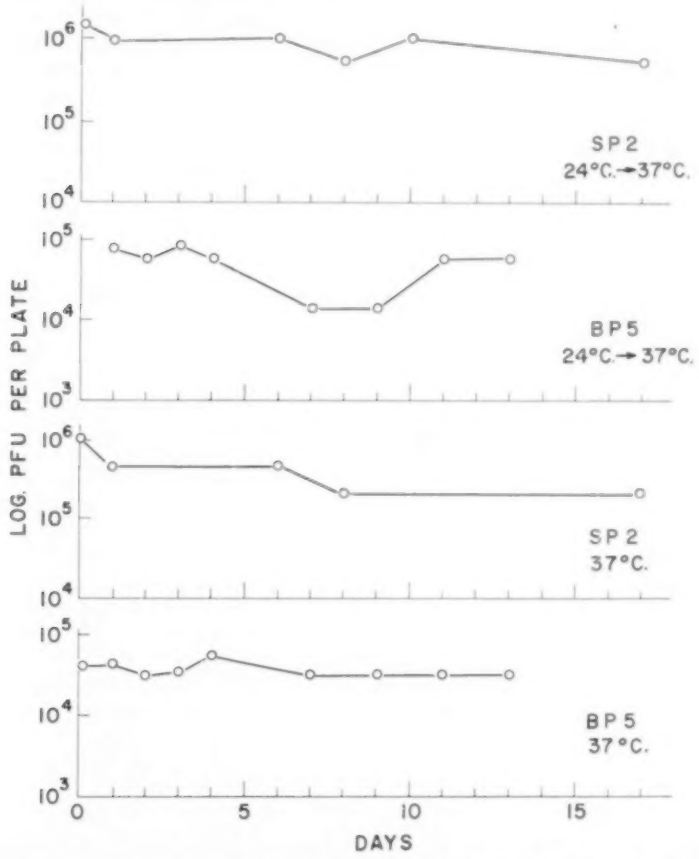

FIG. 5.—Growth curves (total virus) at 24° C. → 37° C., and at 37° C., of polyoma mutants BP5 and SP2 on rat embryo cultures infected at a virus : cell ratio of 3 to 5 PFU per cell. In the 24° C. → 37° C. curves the cultures were maintained for 7 days at 24° C. after infection, and the returned to 37° C. Each curve was determined on cultures made from different embryos. The medium was changed on day 6 for BP5 and day 10 for SP2.

It can be seen from Fig. 6, that even the control cultures of normal cells show only a rise from 6×10^5 PFU to $5{\cdot}3 \times 10^6$ PFU during a period of 10 days, and this is much less than the increase found at this temperature after infection of newly made HE cultures with SP2 (Fig. 4). In addition the transformed and non-challenged cultures in this experiment still contained a very high virus titer, so that no increase in TV content was detectable after challenge (Fig. 6). In

other challenge experiments on cultures with TV contents of 4×10^4 PFU or 3×10^5 PFU taken at 14 and 24 days after infection with BP5, and challenged with BP5 or SP2, there was no virus growth in the controls during a 4 day period at 37° C , and also no increased virus yield after challenge infection of transformed cultures

Cells from 4 hamster primary tumours induced *in vivo*, and from the 6th and 14th transplant generation of an *in vivo* transplanted polyoma induced hamster tumour, also gave no virus growth, during a 4 day period at 37° C., after infection with either BP5 or SP2. These tumours showed no infectious virus production, and tests for inhibitors made with cultures from 3 of the primary tumours by a plaque inhibition test (Winocour and Sachs, 1961*a*), were all negative.

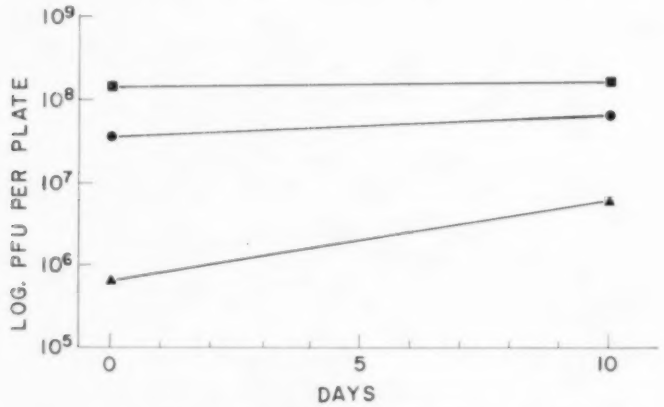

FIG. 6.—Challenge infection (total virus) with polyoma mutant SP2 of hamster embryo cultures transformed by SP2. The controls were normal hamster embryo monolayers of the same age as the transformed cultures. Both the transformed and normal control cultures were infected at 19 days after the original infection, with a virus : cell ratio of 3 PFU per cell, and then maintained at 33° C. for 10 days without a medium change.

■ = Transformed not challenged.
● = Transformed challenged.
▲ = Normal control challenged.

Owing to the absence or very low amount of virus multiplication after infection of the normal older HE cultures, it was thus unfortunately not possible to obtain information on the existence of immunity to superinfection by challenge of mass cultures of transformed cells taken at these short intervals after transformation, or by challenge of cells from *in vivo* induced tumours. The absence of virus growth has also been noted after infection of another polyoma induced tumour transplanted *in vivo*, a culture from normal hamster tissue, (Habel and Silverberg, 1960), and other *in vitro* transformed HE cells observed for 7 days after challenge (Vogt and Dulbecco, 1960).

Experiments on challenge infection with BP5 and SP2 were made with *in vitro* transformed RE cultures, but in accordance with expectation from the results in Fig. 6, there was no virus growth either in the controls or in the challenged transformed cultures.

DISCUSSION

The present experiments have extended our previous observations (Sachs and Medina, 1961), in defining some of the conditions required for the *in vitro* induction of cell transformation and malignancy by polyoma virus. The *in vitro* changes observed after infection of rat and hamster tissues included a fusiform transformation of the cells, and this serves as a useful mark for experimental studies. Other changes observed in transformed cultures included the *in vitro* development of a decrease of contact inhibition, an increased acidity of the infected cultures, a better survival of infected cells under certain nutritional conditions, and a difference in response to trypsinisation. The results with rats indicate that increased acidity associated with transformation does not seem to be due to virus multiplication, since acidity was found in transformed rat cultures without virus growth. The increased acidity in rats and hamsters could be explained in that the metabolism of the transformed cells results in an increased acid production, as in the case of cultures of tumour cells induced *in vivo*.

In tests with tissues other than hamster and rat, an *in vitro* fusiform transformation, similar to that observed with mouse kidney cells (Sachs and Medina, 1961), was found after infection of mouse embryo cells. But no morphological changes were detected after infection of rabbit embryo cells. The apparent lack of such changes in rabbit cells under conditions that produce clear transformation in rat, hamster and mouse cells, may be associated with the fact that the tumours induced *in vivo* by polyoma in rabbits are fibromas that regress (Eddy *et al.*, 1959; Fogel and Sachs, 1960) in contrast to the different types of malignant tumours induced in the other 3 species.

The conditions for *in vitro* transformation that have been studied, included the use of virus mutants and of different temperatures. Of the 3 mutants used, BP5 and IL11 produced large plaques on mouse embryo monolayers and have a PFU : HA ratio of 1×10^5, whereas SP2 produced small plaques and has a PFU : HA ratio of 1×10^6. Virus IL11 (Winocour and Sachs, 1959, 1961*b*) seems to differ *in vivo* from BP5, in that the latter produces in hamsters a very high incidence of early kidney tumours (Axelrad *et al.*, 1960; Stoker, 1960). A comparison of the earliest appearance of morphological transformation after *in vitro* infection of mass cultures of HE and RE cells, showed that this transformation was first observed with SP2, then with IL11, and last with BP5. In experiments with different temperatures there was also an earlier appearance of morphological transformation with BP5 and probably IL11, but not with SP2, if the cultures were kept at 24° C. for 7 days after infection and then returned to 37° C., in comparison to cultures maintained at 37° C. only. Although an earlier transformation, not counting the days at 24° C., was thus observed with some mutants in cultures maintained at 24° C., the low temperature treatment was not essential in order to obtain transformation.

These results thus indicate that there are polyoma mutants with different *in vitro* transforming abilities. These mutants can thus be used for the further elucidation of the process of cell transformation, and other polyoma mutants including SP1 (Medina and Sachs, 1960; Sachs and Medina, 1960) are now being tested. Experiments are also now in progress with single cell clones, in order to elucidate to what extent the difference in the time of appearance of transformation observed after infection of mass cultures is due to a difference in the number

of initially transformed cells, and/or to a difference in the speed of transformation per cell, and whether the morphological transformation is necessarily associated with the decrease of contact inhibition. In addition to plaque size and PFU : HA ratio, a difference in cell transformation would appear to be another useful *in vitro* marker for studies on the genetics of polyoma virus.

It is of interest that morphological transformation with SP2 was observed as early as 2 days after infection of rat and hamster cells and 1 day after infection of mouse cells. These results therefore indicate that cell transformation by polyoma virus can be a rapid event that takes place soon after virus infection.

Experiments on the inoculation of RE and HE monolayers with different amounts of virus, showed that a decrease in the inoculum resulted in an increas in the time of appearance of transformation, and that no transformatiion was observed, even with SP2, when monolayers were incoulated with less than about 1×10^4 PFU per plate. Since these results may be explained in that only 1 cell in about 10^2 was initially transformed after inoculation of the monolayer, and that in addition there is some kind of cell interaction in which a large excess of normal cells prevents cell transformation, this effect of size of inoculum is under further investigation.

Inoculation of transformed cell cultures into adult animals produced progressively growing tumours *in vivo* in 8/42 hamster, and in 0/67 mice. No tumours were formed in 24 hamsters and 34 mice inoculated with uninfected control cultures. The results with hamsters thus show that *in vitro* transformed hamster cells can grow progressively after inoculation into adult hamsters. The results however also show, that not all transformed cultures grew progressively in hamsters, and that none grew progressively in mice. It has been found in all the 3 strains of mice tested (Sachs, 1961*b*) that the growth of readily transplantable tumours can be inhibited by inoculation of the grafted animals with polyoma virus. This demonstrates that the ability of tumour cells to grow progressively after grafting into animals is not only determined by the neoplastic nature of the grafted cells.

All the transformed mouse cultures inoculated into mice contained virus in the inoculum, and this virus would have been sufficient to prevent the *in vivo* growth of tumour cells. The data on the development of hamster tumours in relation to the age of the animal, and the rapid appearance of palpable tumours after inoculation of 18 to 20 day old hamsters (Vogt and Dulbecco, 1960) suggests the possibility that there may be antigenic changes associated with cell transformation which could explain the absence of progressive growth of some cultures after inoculation into adult animals *in vivo*. Thus, although the ability to grow progressively after grafting into animals can be taken as a criterion for tumours, the lack of ability to grow progressively after grafting *in vivo* should not automatically disqualify the transformed cells that do not give successful grafts from inclusion into the category of neoplasms.

In addition to the criterion of progressive growth *in vivo*, it would thus seem desirable to develop criteria for the definition of neoplasia *in vitro*. A decrease of contact inhibition, which has also been observed after *in vitro* transformation with Rous virus (Manaker and Groupe, 1956 ; Temin and Rubin, 1958), and a better survival under certain nutritional conditions, would seem to be satisfactory candidates for inclusion into such a definition.

The experiments on the response of mass cultures of transformed RE and HE

cells to challenge infection, unfortunately did not provide any information on the presence or absence of immunity to challenge infection in cells soon after transformation. In the experiments with rats, there was no virus growth either after infection of normal or after infection of transformed RE cells. In the experiments with hamsters, although good virus growth was found, especially with SP2, after infection of newly made HE cultures, there was no or little virus growth after infection of the older HE cultures that served as controls for the challenge of transformed cells. Single cell clones, picked soon after transformation, are therefore now being examined in the hope of obtaining information on the response of transformed hamster or mouse cells to challenge infection, soon after transformation.

In addition to its potential use in the elucidation of the cell-virus relationship of transformed cells soon after transformation, it seems clear, that the development of a system for the *in vitro* transformation of normal cells by polyoma virus provides a direct approach to the study of the molecular basis of carcinogenesis with a DNA virus under controlled conditions.

SUMMARY

Conditions have been determined for the *in vitro* transformation of rat and hamster cells by polyoma virus. The changes observed after *in vitro* infected included a fusiform cell transformation, a decrease of contact inhibition, an increased acidity of the cultures, a better cell survival under certain nutritional conditions, and a difference in response to trypsinisation. In tests with species other than rat and hamster, the fusiform transformation was found with mouse cells, but not after infection of rabbit cells.

A comparison was made of *in vitro* transformation by 3 polyoma mutants, BP5, IL11, and SP2. The mutants BP5 and IL11 produced large plaques on mouse embryo monolayers and had a PFU :HA ratio of 1×10^5, whereas SP2 produced small plaques and had a PFU : HA ratio of 1×10^6. After inoculation of rat and hamster monolayers, the earliest transformation was observed with SP2, then with IL11, and the latest with BP5. In experiments on transformation at different temperatures, there was an earlier appearance of transformation with BP5 and probably IL11, but not with SP2, if the cultures were kept at 24° C. for 7 days after infection and then returned to 37° C., in comparison to cultures maintained at 37° C. only.

Morphological transformation with SP2 was found as early as 2 days after infection of rat and hamster cells and 1 day after infection of mouse cells. This indicates that cell transformation by polyoma virus can be a rapid event that takes place soon after virus infection.

The results of infection of rat and hamster monolayers with different amounts of virus indicated that a decrease in the inoculum resulted in an increase in the time of appearance of transformation. No transformation was observed in monolayers infected with less than about 1×10^4 PFU per plate of $2{\cdot}5 \times 10^6$ cells.

The growth of *in vitro* transformed hamster cells after inoculation into adult hamsters resulted in the development of progressively growing tumours in 8/42 animals. No tumours developed in hamsters inoculated with control hamster cultures, or in 67 adult mice inoculated with *in vitro* transformed mouse cells. It is however stressed that the *in vivo* development of progressively growing

tumours can be determined by factors other than the neoplastic nature of the cells.

The growth of BP5 and SP2 was studied after infection of normal and transformed rat and hamster cultures. No virus-growth was detected after infection of either normal or transformed rat cells. Although high virus yields were obtained, especially with SP2, after infection of newly made hamster cultures, there was little or no virus growth after infection of older hamster cultures that served as controls for the challenge experiments with transformed cells. The experiments with rat and hamster cultures thus provided no information on the presence or absence of immunity to challenge infection soon after transformation.

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SURVEY OF PAPERS

STOCKS reports an investigation into the association between the high mortality from cancer of the stomach in men in North Wales and previous employment on farms and in quarries (p. 701).

PEDERSEN discusses some factors that might explain the marked dissimilarity in the relative frequency of the various histological types in different groups of cases of lung cancer from Norway, the groups being selected on the basis of how pathological material for histological typing had been obtained (p. 712).

SACKS and TIMME record the autopsy findings in a patient who presented with the clinical and biochemical features of the carcinoid syndrome in association with a metastasising bronchial adenoma (p. 722).

SMITH records the development of reticulum cell sarcoma in two brothers, in each case in the third year of life (p. 731).

JACOBS reviews 93 cases of post-cricoid carcinoma with particular reference to the presence of anaemia and the histology of the epithelium adjacent to the growth. He finds that post-cricoid carcinoma is not usually associated with the mucosal changes found in anaemic patients even when there is a history of anaemia (p. 736).

BLACKLOCK describes the histo-pathological changes which precede the development of the malignant tumours which result from the injection of cigarette smoke condensate directly into the lungs of rats, guinea-pigs and rabbits after thoracotomy (p. 745).

ISRAEL and ELLIS have subjected mice to varying doses of thyroxin deficiency by means of methylthiouracil and a low iodine diet, and have correlated the degree of thyroid hyperplasia with the amount of thyroidectomy cell infiltration of the pituitary. They conclude that even a great excess of thyrotrophic hormone is seldom adequate to produce carcinoma of the thyroid (p. 763).

METCALF finds an increased incidence of lymphoid leukaemia, plasma cell tumours and reticulum cell sarcomas in C3H mice subjected to weekly antigenic stimulation for life (p. 769).

LLOYD reports a sudden rise in the incidence of squamous cell carcinoma of the ear, muzzle and perineum in a flock of Merino sheep. Attention is drawn to the occurrence of photosensitisation in the flock preceding the rise in incidence and its possible role as a promoting agent is discussed (p. 780).

CHESTERMAN and NEGRONI find that the injection of large amounts of polyoma virus (Mill Hill strain) produces in hamsters a variety of sarcomas, vascular lesions, or both, and that these begin to appear in the kidney as early as the third day after inoculation (p. 790).

VAN DEN BRENK has determined the number of Ehrlich ascites tumour cells required to induce 50 per cent of single tumours in mice for both single (one leg) and double (two leg) inoculations, and similarly for the induction of double tumours resulting from double inoculations. These findings have been compared with the corresponding cell doses required after immunological depression of host mice with whole body X-irradiation (p. 798).

SEARLE has confirmed the carcinogenicity of β-propiolactone for mouse skin, and has examined its reaction with cysteine and cystine polarographically. The destruction of the cysteine catalytic wave in cobaltous buffer was in accord with the formation of S-2-carb-

oxyethylcysteine, contrasting with the effects observed on the catalytic double wave of cyst(e)ine in protein combination. No tumours were induced in mouse skin by repeated application of 3-bromopropionic acid (p. 804).

Schoental finds no evidence of pyrrolizidine alkaloids in a batch of MRC Diet 18 which proved toxic to guinea-pigs. The toxicity appears to be related to its content of ground nut meal (p. 812).

Ray, Cromeɴ, Aycock and Pitzer have studied the gastric secretion of two basic carcinogens, 2-acetylaminofluorene and 2,7-diacetylaminofluorene in the rat. The latter compound, which alone causes gastric adenocarcinoma in this species, was taken up by the stomach wall to a greater extent than the former (p. 816).

Marchant has studied the effect of hypophysectomy on the induction of ovarian tumours in mice treated with DMBA. She finds that although DMBA proves toxic to hypophysectomised mice, the operation does not prevent DMBA treatment from rendering ovaries preneoplastic. Such ovaries can develop into tumours when grafted into hosts with intact pituitaries, but preneoplastic ovaries grafted from DMBA-treated into hypophysectomised mice fail to develop into tumours (p. 821).

Daniel and Prichard report the production of mammary tumours in 149 out of 163 female rats of the Sprague-Dawley strain by the oral administration of 3-methylcholanthrene (p. 828).

Goodlad and Clark find that the inactivation of corticosterone by Ring A reduction in livers of rats bearing the Walker 256 carcinoma is increased at an early stage in tumour growth. This finding is discussed in relation to the accompanying adrenal hypertrophy (p. 833).

Lundin has studied the carcinogenic actions of three different iron complexes injected intramuscularly into rats. Two of the preparations investigated induced a high incidence of sarcomas whereas the third produced only a single tumour, apparently a fibroma (p. 838).

Colella, Reith and Williams have extended the ^{131}I-triolein fat absorption test to study the blood radioactivity levels in patients with malignant disease. They find the blood radioactivity after ingestion of the labelled fat to be lower in cancer patients than in normal controls, and that successful treatment moves the curve of blood radioactivity towards the normal range (p. 848).

Calcutt finds that neither the timing nor the extent of rises in mouse skin —SH levels induced by cocarcinogens is influenced by previous treatment with a carcinogen (p. 855).

Adams, Rowson and Salaman report on a transmissible agent which produces a rapid rise in plasma lactic dehydrogenase activity when injected into mice and which is found in the plasma of mice bearing homologously transplanted tumours, but not in that of mice with spontaneous leukaemia (p. 860).

Whitaker has studied the plasma β-glucuronidase in 41 cases of breast carcinoma. He finds that oral methyl testosterone, ethinyl oestradiol and stilboestrol raise the enzyme; intramuscular testosterone, non-virilizing androgens and cortisone have no effect. Cases responding to hypophysectomy have lower pre-operative titres than those which do not (p. 868).

Medina and Sachs have determined the conditions for the *in vitro* induction of cell transformation and malignancy after infection with polyoma virus, including the use of virus mutants, and the growth of polyoma virus has been studied after infection of normal or *in vitro* transformed hamster and rat cells (p. 885).

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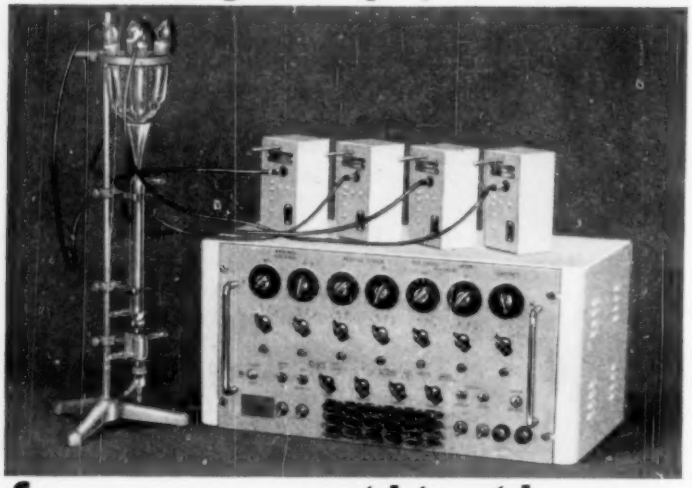

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